Endocrine Studies in the Premenstrual Syndrome.

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ENDOCRINE STUDIES IN THE PREMENSTRUAL SYNDROME

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A thesis submitted for the degree
of Doctor of Philosophy

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March 1979

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ABSTRACT

A possible hormonal basis for the premenstrual syndrome (PMS) was tested by measurement of progesterone, oestradiol and aldosterone throughout the menstrual cycle in subjects with PMS and in controls, in plasma.

An antiserum was raised against progesterone-11α- hemi-succinate-BSA in rabbits and a radioimmunoassay developed, and oestradiol was assayed after extraction using a specific antiserum. Sephadex LH-20 microcolumns were used to purify aldosterone from plasma extracts before radioimmunoassay. The sensitivity, precision, accuracy and specificity of all the assays were found to be satisfactory.

When ten control and 20 PMS cycles were compared, it was found that the midluteal peak of progesterone (from 8 to 5 days premenstrually) was lower ($P < 0.02$), and plasma oestradiol levels were higher in the last 4 days of the cycle ($P < 0.01$), in the PMS group.

The pattern of aldosterone in the menstrual cycle of non-fasting controls showed higher levels after ovulation but there was not a pronounced pre-ovulatory peak. There were no marked differences between the PMS and control groups.

When a further 90 subjects with PMS were studied, a third of these had progesterone levels below the 90 per cent confidence limits of the controls. There was an inverse relationship between progesterone and serum prolactin levels in the luteal phase. Women complaining chiefly of physical symptoms, such as water retention, had higher progesterone levels than those complaining chiefly of irritability and depression, but progesterone levels did not correlate with psychiatric ill-health.

In a clinical trial of dydrogesterone, it was shown that progesterone levels were slightly reduced in the dydrogesterone-treated cycles despite clinical improvement.

These results support the concept that endocrine imbalance may occur in some PMS patients but not all. The aetiology of the various forms of the syndrome is still obscure but possible mechanisms are discussed.

ACKNOWLEDGEMENTS

This work was carried out in the Department of Gynaecology, St. Thomas's Hospital Medical School.

I would like to thank Dr. M. G. Brush for his encouragement and advice during the supervision and discussion of this work. I am also grateful to the clinical members of the Department for referring the patients and to other colleagues, particularly Mrs J. Judah, for help with the blood samples.

I am greatly indebted to Dr. M. J. Wheeler and his staff in the Department of Chemical Pathology for the prolactin assays.

I also wish to acknowledge the assistance of Dr. S. C. Darby of the Department of Community Medicine with the computer programme, and of Dr. A.W. Clare, Institute of Psychiatry, for the psychiatric assessments.

I am also grateful to my family for their extreme patience and tolerance during the preparation of this thesis.

This work was supported by the St. Thomas's Hospital Charitable Trust and the St. Thomas's Hospital Endowment Funds.

This study would not have been possible without the cooperation and interest of the patients and volunteers.

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CHAPTER 1

Review of the literature

1. THE MENSTRUAL CYCLE

There are many examples of biological processes which are rhythmic or cyclical in character, and the human menstrual cycle is perhaps one of the most complex. Therefore, to investigate a disorder such as the premenstrual syndrome, it is necessary to understand processes in the 'normal'cycle.

External factors affecting the cycle are not well understood, although such factors as the moon (Menaker & Menaker, 1959) and pheromones (McClintock, 1971) have been mentioned.

Several authors have attempted to provide definitive figures for the average menstrual cycle, for instance Chiazze,Brayer, Macisco, Parker & Duffy (1968) in a study of at least ten cycles each from over 2000 women found that the average cycle length per woman was 29.1±7.5 days. Other estimates of the average length of the human cycle have varied from 27.3 to 31.9 days (Presser, 1974). Differences are probably due to differences in the samples of women, especially with regard to age. In one longitudinal study (Treloar, Boynton, Behn, & Brown, 1967) it was found that cycles were longer and more variable in length in the post menarche and premenopausal years, although cycles tended to get progressively shorter from the twenties to the forties.

The two reference points of the cycle are ovulation and menstruation, and it is the post-ovulatory (luteal) phase which is most constant (Presser, 1974).

The intrinsic events of the cycle have been investigated fairly thoroughly, especially the endocrinological and physiological aspects, although the social and psychological aspects are rather more controversial.

A review of all these events will be undertaken in the

hope of throwing some light on the aetiology of the premenstrual syndrome.

1.1. HORMONAL CHANGES IN THE CYCLE

1.1.1. The control of ovulation

The most important processes of the cycle, from the evolutionary point of view, are those that control ovulation and facilitate implantation. These events are under the control of the hypothalamus, pituitary and ovary, with interactions between the rest of the central nervous system. The individual hormones have a characteristic pattern of secretion and it is important to establish these patterns before causative relationships can be worked out, especially in humans where experimental studies are more difficult.

Present knowledge of hormonal events comes largely from radioimmunoassay data, obtained in the last ten or so years, although earlier work established the basic patterns.

Gonadotrophic releasing factor

Gonadotrophin releasing factor, GRF (or gonadotrophin releasing hormone) is a decapeptide released by the hypothalamus with actions on the pituitary release of luteinising hormone (LH) and follicle stimulating hormone(FSH) (Schally, Arimura, Kastin, Matsuo, Baba, Redding, Nair, Debeljuk,& White, 1971). Semiquantitative bioassay methods have been used to measure levels in peripheral blood, and a small peak was found at midcycle, at about the time of ovulation (Malacara, Seyler & Reichlin, 1972). This was confirmed by radioimmunoassay, and GRF immunoreactivity was only consistently detectable at midcycle (Arimura, Kastin, Schally, Saito, Kumasaka, Yaoi, Nishi & Okhura , 1974). This probably represents a 'leak' of the hormone into peripheral blood since GRF is stored in the median eminence (Crighton, Scheider & McCann) and is secreted directly

to the pituitary via the portal system.

The Gonadotrophins

Patterns of FSH and LH have been extensively studied, firstly in urine and later in serum. McArthur, Worcester, & Ingersoll (1958), using a specific bioassay, the ventral prostate test, first demonstrated that a peak in LH excretion occurred at day 16 of the cycle. The first report of FSH excretion used a specific ovarian augmentation assay in mice (Brown, 1955). Holmstrom & Salhanik (1956) showed high levels during menses in their small group of women, but changes in the rest of the cycle were inconsistent. Fukushima, Stevens, Gantt & Vorys (1964), using a different bioassay, established that a second rise always occurred around midcycle. These results were confirmed by many bioassay and some immunological assay methods for LH (Brown, Klopper & Lorraine, 1958), and FSH (Persson & McCormick, 1968), although the data obtained were limited by the problem of sensitivity. However, an accurate picture of temporal events was made possible by radioimmunoassay methods which gave adequate sensitivity and specificity in urine and blood. Bagshawe, Wilde & Orr (1966) showed that both urinary and serum LH reached a sharp peak at midcycle, with low levels throughout the rest of the cycle, and this was confirmed by others (Odell, Ross & Rayford 1967; Stevens, 1969). It was also confirmed that FSH had two peaks in the cycle, one early in the follicular phase and a second at or near ovulation, and that the midcycle peaks of LH and FSH coincided (Midgley & Jaffe, 1968, Faiman & Ryan, 1967). Other workers have since reported gonadotrophin levels together with other hormones in the normal menstrual cycle, and these will be discussed below.

The ovarian steroids

Oestrogens

The individual oestrogens oestradiol, oestriol and

oestrone was first measured in urine in the classic studies of Brown (1955a). The methodology is discussed on page 60 . Brown found that all three oestrogens were very low at the beginning of the cycle but rose to a peak at midcycle. They then fell sharply but rose to a second, smaller peak in the luteal phase, falling again in the last few days of the cycle before menstruation. Oestradiol was present in smaller amounts than oestrone and oestriol, but the total oestrogen output could be as little as 5 µg/24 hours. Baird & Guevara (1969) reported the first levels in blood, using a double isotope method, and correlated oestrone and oestradiol levels with LH. They found that oestradiol levels started to rise before any rise in LH, and postulated that another trophic factor was responsible.

Oestradiol is thought to be the most important oestrogen produced by the follicles, since it occurs at greater concentration than oestrone in follicular fluid (Smith & Ryan, 1962) and is the major radioactive metabolite of ^{14}C-acetate preincubated with follicles or corpora lutea (Ryan & Smith, 1961) . The ratio of oestradiol to oestrone shows a significant predominance of oestradiol during the pre-ovulatory period(Baird & Guevara, 1969). Therefore oestradiol has been regarded as the most important oestrogen to study in the menstrual cycle and since the technique of radioimmunoassay (RIA) several studies of daily levels have been carried out. All have shown the midcycle peak and a secondary oestradiol peak in the luteal phase(Abraham, Swerdloff, Tulchinsky, Hopper & Odell, 1971; Mikhail, Wu, Ferin & Vande Wiele, 1970).

Progesterone

The first general indication of progesterone secretion came from measurement of its urinary metabolite, pregnanediol. Klopper (1957), using a chemical method carried out the first reliable clinical measurements. He found that pregnanediol excretion was very

low throughout the follicular phase, at less than one milligram daily. It began to rise after ovulation, reaching a peak of between 2 and 5 mg. daily at about day 21 of the cycle. Levels then fell several days before menstruation and continued to decline for a few days after its onset. Klopper (1957) pointed out the wide variability in the levels of pregnanediol in the luteal peak.

Plasma progesterone was found to follow the same pattern as its urinary metabolite, with tenfold increases in the luteal compared to the follicular phases, when double isotope or electron capture GLC methods were used (see p.58).

The development of competitive protein binding assays and RIA enabled repetitive sampling of plasma throughout the cycle (Neill, Johansson, Datta & Knobil, 1967; Yoshimi & Lipsett, 1968), where the same pattern was seen.

Other Ovarian Steroids

Competitive protein binding (CPB) and RIA techniques have also been applied to the measurement of 17α-OH progesterone (Strott, Yoshimi, Ross & Lipsett, 1969; Abraham, Swerdloff, Tulchinsky, Hopper & Odell, 1971). This progestogen shows a rise parallel to progesterone in the luteal phase and also a midcycle peak, coinciding with the LH peak.

Plasma pregnenolone shows a slight tendency to rise in the luteal phase but this only becomes a significant difference when values are pooled for six subjects (Abraham, Buster, Kyle, Corrale & Teller, 1973). Pregnenolone is the precursor for both the ovarian and the adrenal cortical steroids, so it is possible that the ovarian pattern may be masked by metabolism occurring in the adrenal (see Fig 6.1)

Another progestogen studied is 20α-dihydroprogesterone. While the values for this steroid and progesterone are about the same in the follicular phase (less than 2 ng/ml) and they both reach a

peak in the luteal phase, the luteal values for 20∝-dihydro-progesterone are about one fifth of those of progesterone (Florensa, Sommerville, Harrison, Johnson & Youssefnajadian, 1976).

Hormonal relationships in the control of ovulation

An integration of all the hormonal events of the cycle was finally accomplished in the early 1970s by daily measurements of all the hormonal components in individual women. The lengths of the cycles have been standardised using the day of the LH peak as day 0. Studies were carried out by Ross, Cargille, Lipsett, Rayford, Marshall, Strott & Rodbard (1970) in 16 cycles, by Dyrenfurth, Jewelewicz, Warren, Ferin & Vande Wiele (1974) for 40 cycles and by Mishell, Nakamura, Crosignani, Stone, Kharma, Nagata & Thorneycroft (1971) for 10 cycles.

An example of the composite pattern is shown in Fig.1.1. From these composite graphs events leading up to ovulation can be clearly seen. From the onset of bleeding FSH levels are raised while oestradiol is very low, until about day 7. During the following week or so the oestrogens rise slowly and then rapidly, reaching a midcycle peak, while FSH declines. Thus FSH is the trophic hormone leading to development of the follicles while oestradiol inhibits FSH secretion by negative feedback.

The oestradiol peak at midcycle occurs marginally before the gonadotrophin peaks, and it appears that the oestradiol peak triggers a surge of LH and a smaller surge of FSH. It appears that changes in gonadotrophin secretion are brought about by changes in pituitary responsiveness to LRF (Nillius, Friberg & Wide, 1973). Exogenous oestrogens have been shown to enhance pituitary sensitivity to LRH (Shaw, Butt & London, 1975). This rather complex action of oestrogens on gonadotrophin release, with negative and then positive feedback, has been demonstrated experimentally in ovariectomised women (Yen, Vandenberg, Tsai & Siler, 1974).

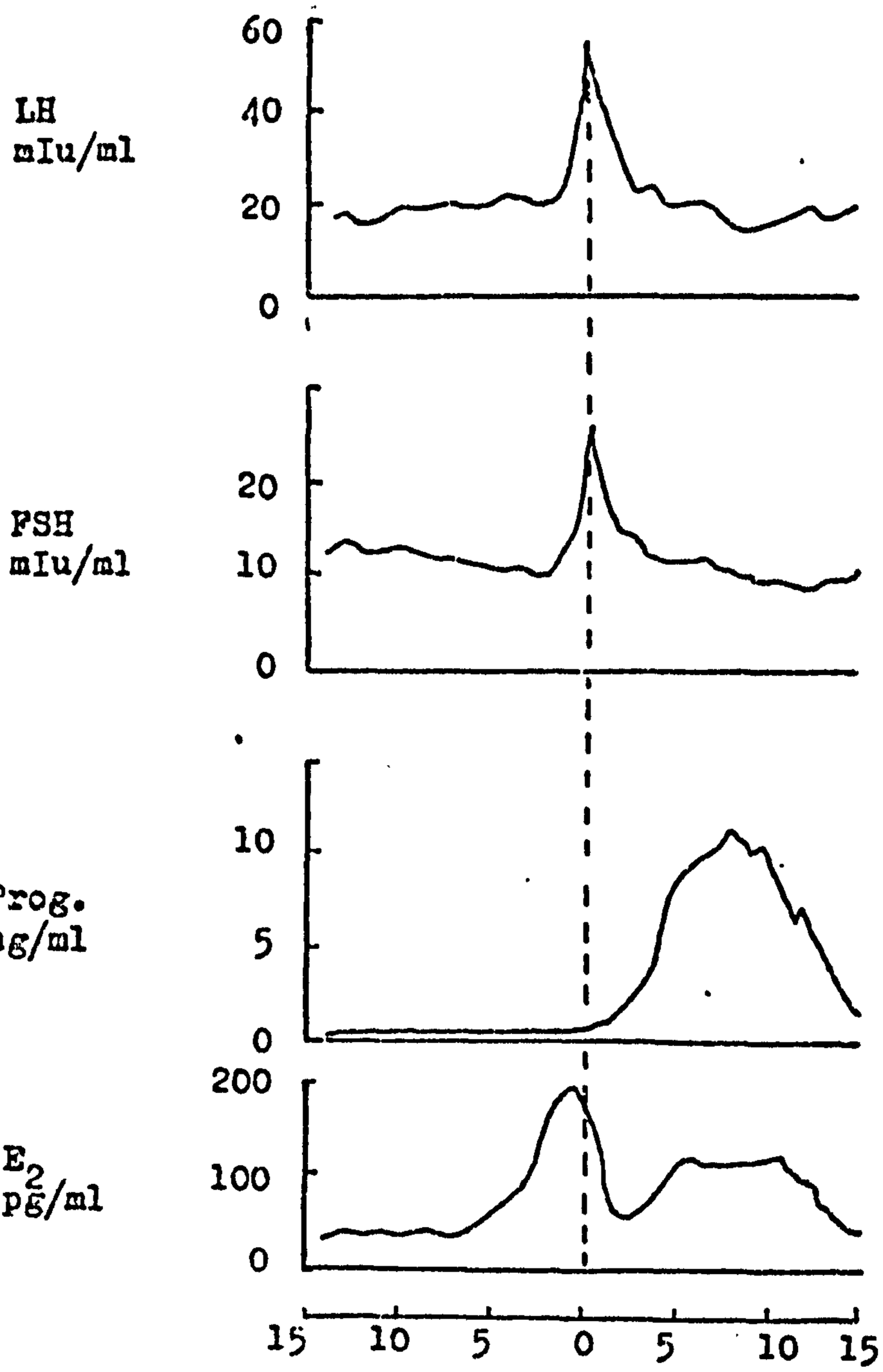


Figure 1.1. Profiles of serum LH, FSH, progesterone (prog) and oestradiol (E_2) during ten ovulatory cycles. The day of the LH maximum value is the reference day. (From Mishell et al, 1971)

Once the LH peak occurs, ovulation follows (Yussman & Taymor, 1970) and a corpus luteum is formed, which produces progesterone, and to a lesser extent oestrogens and other progestogens. The role of progesterone in the LH surge is controversial. There is evidence that progesterone starts to rise 16 hours before ovulation (Yussman & Taymor, 1970), but that the LH peak precedes the rise in progesterone by 12 hours (Johansson & Wide, 1969). However there is a recent indication using RIA that a modest rise in progesterone occurs a day or two before the LH peak (Florensa et al., 1976). This suggests that progesterone is secreted by the Graafian follicle or other ovarian tissue before the LH peak and there is some indication that this progesterone may augment pituitary responsiveness to LRF (Nillius & Wide, 1976).

During the week after the LH peak steroid production by the corpus luteum increases, reaching a peak and then declining during the second postovulatory week. Unfortunately the exact temporal relationship of the decline in steroid secretion and the onset of menses cannot be determined from these composite cycles. This is due to practice of taking the LH peak as the reference point. However in papers where hormones from individuals are shown, (Abraham et al., 1971a; Dyrenfurth et al., 1974), it appears that both progesterone and oestradiol are very low at menstruation, although Yoshimi & Lipsett (1968) noted that in 2 out of 3 of their subjects, progesterone was still elevated at the onset of menses.

Many luteolytic and luteotrophic substances have been found in animals (Short, 1964) but in humans the situation is less clear. The gonadotrophins are very low in the luteal phase, suggesting that the corpus luteum is independent of the pituitary, yet hypophysectomised women require small amounts of LH to maintain corpus luteal function (Vande Wiele, Bogumil, Dyrenfurth, Ferin, Jewelewicz, Warren, Rizkallah & Mikhail, 1970). Similarly if HCG

is administered during the luteal phase the production of progesterone is increased in normal women (Strott et al., 1969).

The cycle starts again as the corpus luteum regresses, the menses occur, and FSH levels begin to rise. This effect is, again, not clearly seen in composite cycles but can be seen in individuals (Abraham et al., 1971a).

Thus a very complex and precise control is shown in the normal menstrual cycle, but it should be emphasised that considerable variations occur in individual hormone patterns without apparent pathology. It appears that rapid cycles of hormone release are superimposed onto the much longer menstrual cycle. For instance LH has a pulsatile secretion with a periodicity of 90 minutes and oestrogens affect the amplitude of these pulses (Yen, Vandenberg, Tsai & Parker, 1974).

Thus the control of ovulation can be seen as continuous adjustment of rapid fluctuations, probably with external factors acting via the CNS to alter pulsatile secretions from the pituitary. The role of the hypothalamic monoamines in pituitary function has been studied in the rat and other animals. Although the evidence is inconclusive, it appears that dopaminergic pathways are inhibitory while pathways involving serotonin stimulate gonadotrophin release (for review see Smythe, 1977). In human subjects serum LH levels are significantly suppressed after the infusion of dopamine (Leblanc, Lachelin, Abu-Fadil & Yen, 1976).

Another group of compounds with possible effects on gonadotrophin release are the prostaglandins, since in rats prostaglandin E_2 can stimulate LH release(Harms, Ojeda & McCann, 1973).

1.1.2 The renin-angiotensin-aldosterone system

There is evidence that the mineralocorticoid aldosterone, which is produced by the adrenal cortex, also has a cyclical pattern of secretion. The major role of aldosterone is to maintain sodium homeostasis, and the mechanism whereby sodium depletion activates aldosterone secretion is mediated by factors such as renin substrate, renin enzyme, which occurs in the kidney, and angiotensin II.

Reich (1962) first looked at urinary levels of aldosterone in six women and found that a characteristic excretory pattern occurred. Although variation between individuals was very great, increased aldosterone excretion was seen at the midcycle, and a second peak was noted at one to five days before menstruation.

A study of aldosterone secretion rates was carried out by Gray, Strausfeld, Watanabe, Sims & Solomon (1968). They compared the mid-follicular and mid-luteal secretion rates and found a twofold increase in the second half of the cycle. Sundsfjord & Aakvaag (1972) obtained similar results for the urinary excretion of aldosterone and they also found increased plasma renin activity in the second half of the cycle. They did not find any significant change in plasma renin substrate.

Studies of plasma aldosterone levels were undertaken when radio-immunoassay methods became available. Blood samples from supine patients on controlled diets were taken throughout the cycle in several studies (Katz & Romfh, 1972, Michelakis, Yoshida & Dormois, 1975). In both these papers a cyclical pattern is seen with higher values in the luteal phase but no clear-cut preovulatory peak. In a recent paper, however, Frölich, Brand, & van Hall (1976) studied non-fasting ambulant patients and found an additional peak of plasma aldosterone on the day prior to ovulation, which is in agreement with an earlier study of aldosterone plasma levels (Sundsfjord & Aakvaag, 1973).

The factors underlying the cyclical changes in the renin-angiotensin-aldosterone (RAA) system are not yet understood. Possible

direct mechanisms include an increase in glomerular filtration rate, or an exaggeration of the diurnal translocation of fluid which occurs on the assumption of upright posture. The first suggestion is unlikely since Gray et al. (1968) could find no increase in creatinine clearance during the secretory phase peak of aldosterone. The second idea has not been fully resolved in this context, although an interesting study was carried out by Edwards & Bayliss (1973). They measured water and electrolyte excretion in the menstrual cycle and found no difference throughout the cycle in supine women. However, on tilting the subjects upright they found decreased urine volume, sodium and potassium excretion, and this effect was enhanced in the luteal phase. They speculate that the changes with posture were due to an alteration in proximal tubular function, an effect which would be heightened in the luteal phase. However differences in proximal tubular function do not account at present for all the cyclical changes seen, since Katz & Romfh (1972) and Michelakis et al (1975) found a luteal increase in plasma aldosterone even in supine women. The effect of posture is not fully understood and requires further work.

Other components of the RAA system have been studied. Katz & Romfh (1972) found that while plasma aldosterone and renin activity fluctuated in parallel, there was no cyclical pattern of renin substrate concentration. This is in agreement with Sundsfjord & Aakvaag (1972). There is also evidence that plasma angiotensin II is elevated in the second half of the cycle (Sundsfjord & Aakvaag, 1970).

Thus it appears that the increase in aldosterone is brought about by increased renal production of renin, at least in part. It is possible that the cyclical pattern of the steroids oestradiol and progesterone may be responsible for this. It has been shown that the luteal rise in aldosterone is abolished in women taking oral contraceptives (Gray et al, 1968) and in women judged to have luteal failure (Sundsfjord & Aakvaag; 1972).

The role of the oestrogens is uncertain. Sundsfjord & Aakvaag (1972) studied the hormones of women with normal cycles and those with luteal failure. In the latter the luteal peak of progesterone was abolished, with progesteronevalues of 3ng /ml or less, but oestrogen levels in the luteal phase of these women were the same as the normal group. However, they found that the luteal peak of aldosterone was also abolished. They concluded from this that the oestrogens are not responsible for the premenstrual changes in the RAA system.Laidlaw, Ruse & Gornall (1962) had earlier found that physiological doses of oestrogens had no effect on aldosterone excretion.

Yet other workers have shown that ethinyloestradiol increases aldosterone excretion (Crane & Harris, 1969), and renin activity (Kaulhausen, Muhlbauer, Beck & Breuer, 1974), both of which rise in parallel with oestradiol in the luteal phase. However ethinyl oestradiol also increases plasma renin substrate, (Pallas, Holzwarth,Stern & Lucas, 1977) and this does not alter cyclically. Perhaps there are nonparallel effects in different parts of the RAA system. In addition the effect of synthetic oestrogens such as ethinyl oestradiol must be interpreted with caution.

The role of progesterone is more clear-cut. Progesterone has natriuretic effects in man at physiological doses and inhibits aldosterone at the renal level. This leads to increased aldosterone secretion which will overcome the natriuresis after 3 to 8 days.(Landau, 1973). Sundsfjord & Aakvaag (1972) considered that the luteal peak of aldosterone was abolished in their luteal failure group as a direct result of the low progesterone values. In studies of the effects of progesterone, Laidlaw et al (1962) found that physiological doses of progesterone increased aldosterone excretion, while Oelkers, Schöneshöfer & Blümel (1974) found an increase in plasma renin activity, plasma angiotensin II, and aldosterone excretion, but plasma renin substrate concentration remained unchanged

Thus the luteal peak of progesterone may fully account for the changes seen in the RAA system at that time. Since no consistent changes occur in urine volume, sodium and potassium excretion in the luteal phase (Gray et al. 1968), it appears that in most circumstances the diuresis and natriuresis brought about by progesterone is compensated for by the salt-retaining effect of increased aldosterone secretion. However Gray et al (1968) could find no consistent relationship between secretion of aldosterone and urinary pregnanediol, in contrast to the findings of Jones, Lloyd-Jones, Riondel, Tait, Tait, Bulbrook & Greenwood (1959) who obtained a good correlation in pregnancy. The work of Gray et al (1968) has been criticised on the grounds that the salt intake of all the subjects was not controlled (Landau, 1973).

1.1.3. Prolactin

In animals serum prolactin follows a marked pattern of secretion throughout the oestrus cycle (Neill & Reichart, 1971). In women, prolactin has been measured throughout the cycle by several investigators but no agreement has been reached as to whether a cyclical pattern exists.

McNeilly & Chard (1974) took daily blood samples from eight women with regular cycles, and they also studied the variability of prolactin at 15 minute intervals at different stages of the cycle. They found a small but nonsignificant rise in serum prolactin at midcycle, associated with the peaks of LH and FSH. They could find no relationship between prolactin and oestradiol or progesterone. They noted that serum prolactin showed considerable fluctuations, apparently at random, and also considerable differences between individuals. Other workers have found similar results but with increased luteal levels (Franchimont, Dourcy, Legros, Reuter, Vrindts-Gevaert, Van Cauwenberger & Gaspard,1976). Vekemans, Delvoye, L'Hermite & Robyn (1977) studied serum prolactin in 34 cycles and found a progressive increase in serum prolactin during the follicular phase to reach a peak at the same time as LH. A second peak of prolactin was seen in the luteal phase. They studied an additional 17

cycles using a slightly different assay method and obtained slightly different results, with a sharper peak at ovulation and a less pronounced luteal rise. Unfortunately it is impossible to say whether this discrepancy is due to true differences in the method or between the two groups of women.

These papers serve to show the difficulties in obtaining definitive results for prolactin, especially in view of the extreme lability of the hormone (Robyn, Delvoye, Nokin, Vekemans, Badawi, Perez-Lopez & L'Hermité, 1973) and the effect of non-specific factors such as stress (Noel, Suh & Frantz, 1971). Vekemans et al (1977) found that differences between stages of the cycle were quite small, with only a 50% increase at the most from early follicular levels, yet random changes in one day may be as great as 110% (McNeilly & Chard, 1974). Therefore it is difficult to see what role serum prolactin may play in the events of the menstrual cycle.

Indeed, when prolactin secretion is suppressed by bromocryptine, a specific inhibitor, no effect is seen on LH, oestradiol, or progesterone in normal women (del Pozo, Goldstein, Friesen, Brun del Re & Eppenberger, 1975). Conversely, some cases of high prolactin levels lead to anovulation and amenorrhoea, and this is now well documented (Besser, Parke, Edwards, Forsyth & McNeilly, 1972; Pepperall, Evans, Brown, Smith, Healy & Burger, 1977). It has been shown that high prolactin levels abolish the pulsatile secretion of LH, suggesting that prolactin is acting at the hypothalamic or pituitary level (Bohnet, Dahlen & Schneider, 1974).

Although prolactin at normal physiological levels does not appear to influence ovulation, there is a possible role at the local level of the Graafian follicle. McNatty, Sawers & McNeilly (1974) studied human granulosa cells in culture. The cells were cultured for up to 14 days and progesterone production was studied after prolactin was added to the medium. Prolactin concentrations of up to 20ng/ml permitted increasing progesterone

synthesis but above this progesterone secretion was inhibited. They also found that endogenous prolactin levels were lowest in the follicles in the late follicular and early luteal phase.

The changing hormones of the menstrual cycle may in turn influence prolactin secretion. It has been shown by Robyn et al (1973) that high doses of exogenous oestradiol lead to increased serum prolactin in normal women. The administration of progestogens had no effect.

1.1.4. Other hormones

It has recently been shown by Frölich et al (1976) that the serum levels of dehydroepiandrosterone do not show a well defined cyclical pattern. However androstenedione, testosterone and aetiocholanolone levels are raised during the ovulatory phase (3 days before ovulation until 4 days after).

There is a statistical difference in plasma cortisol concentrations between the first and second halves of the cycle, with lower values at day 24 compared to day 10 (Beck, Morcos, Fawcett, & Watanabe, 1972). However this only holds true for morning levels of cortisol. West, Mahajan, Chavre, Nabors & Tyler (1973) could find no cyclic variation in cortisol levels.

No consistent changes have been reported for the protein hormones such as thyroxine (Weeke & Hansen, 1975), although fasting blood sugar and glucose tolerance are altered at menstruation (Southam & Gonzaga, 1965). A recent study (Saxena, Dusitsin, & Lazarus, 1974) could find no cyclical pattern for human growth hormone, thyroid stimulating hormone, or cortisol in Thai women.

1.1.5 Catecholamines

Another interesting group of substances are the catecholamines such as adrenaline, noradrenaline and dopamine (which is also prolactin inhibitory factor). Although there is little direct evidence that these neurohormones themselves vary throughout human menstrual cycles, there is indirect evidence from metabolic pathways.

The monoamine oxidases are enzymes involved in the degradation of the biologically active amines such as 5-hydroxytryptamine (5HT) and dopamine.

Holzbauer & Youdim (1973) studied MAO activity in the rat uterus, ovaries, adrenals, and in four regions of the brain, and found higher values at diestrus than at metestrus or estrus. In humans, it has been shown that cyclical variations occur in endometrial MAO (Southgate, Grant, Pollard, Pryse-Davies & Sandler, 1968), with higher levels in the late secretory phase. This suggests that MAO activity may be hormonally dependent. Yet in a study of platelet and plasma MAO (Gilmore, Robinson, Nies, Sylvester & Ravaris, 1971) no significant difference was found between midcycle and premenstrual values. A more recent paper (Belmaker, Murphy, Wyatt & Loriaux, 1974) has found that a cyclical pattern in platelet MAO does exist, with a decline in the luteal phase. In contrast, Klaiber, Kobayashi, Broverman & Hall (1971) found an increase in plasma MAO in the luteal phase. Platelet and plasma MAOs appear to be distinct proteins, with different responses to inhibitors (Robinson, Lovenberg, Keiser & Sjoerdsma, 1968).

1.2 PHYSIOLOGICAL CHANGES IN THE CYCLE

The best known event occurring cyclically is the increase in basal body temperature which is seen after ovulation and is a fairly reliable indication of progesterone secretion by the corpus luteum (Ross et al, 1970).

There is a significant rise in heart rate and respiration rate and a fall in sweat gland activity in the luteal phase (Little & Zahn, 1974). These changes may be associated with the rise in body temperature and ultimately with the thermogenic effect of progesterone, which has been known for many years (Barton & Wiesner, 1945).

Abramson & Torghele (1961) also found a consistent positive

relationship between temperature and daily body weight in a study of 34 self-selected students. They found peaks in weight at around the early follicular (days 3-6) ovulation (days 13-15) and the premenstrual phase (days 24-26) and variations of up to 10 pounds were seen. In a similar study Golub, Menduke & Conly (1965) could find only negligible weight gain in the cycle (less than one pound) and this occurred post-menstrually. The differences in these two papers may partly reflect the different methods used by these authors to standardise the length and stages of the menstrual cycle.

In a controlled study using psychiatric in-patients complaining of premenstrual tension Bruce & Russell (1962) could find no weight gain if the patients were on an uncontrolled diet. However, in a metabolic ward where diet was controlled, a slight weight gain (less than 500 grams) was seen in the last five days of the cycle before menstruation, and a sharp rise in weight occurred at ovulation.

Water and electrolyte excretion has been measured in both the follicular and luteal phase of the cycle in subjects undergoing prolonged diuresis (Edwards & Bayliss, 1973). They found no difference when patients were supine but statistical differences occurred in up-right patients, with lower urine volume and electrolyte excretion in the luteal phase. Gray et al (1968) could find no change in total body water, serum sodium and the sodium/potassium ratio in ambulant subjects, and this has been confirmed by Michelakis, Stant & Brill (1971) in patients with constant salt intake.

There is some evidence that human uterine fluid potassium is increased in the luteal phase (Clemetson, Kim, de Jesus, Mallikarjuneswara, & Wilds, 1973), and this may be an indication of other tissue changes pertinent to the problem of cyclical oedema. However, the uterus may be a special case since it is a target tissue for the ovarian steroids. While

there are changes in prostaglandin levels in the endometrium, reports vary as to whether a cyclical pattern exists in blood (Karim & Hillier, 1975).

Premenstrual hyperemia of the breasts has been noted for many years (Taylor, 1949) and increase in breast volume also occurs in the second half of the cycle (Milligan, Drife & Short, 1975), which may have some bearing on premenstrual breast discomfort. There is also a report that progesterone causes smooth muscle relaxation, in ureter, bowel and stomach (Kumar, 1962), and this may be related to abdominal bloatedness, which may be due to the laxity of the anterior abdominal wall (Dalton, 1977).

Many other changes occur in blood properties which are related probably to menstruation itself (such as erythrocyte count)and the review of Southam & Gonzaga (1965) is still the most extensive account of these and other changes.

1.3.BEHAVIOURAL CHANGES IN THE CYCLE

The psychophysiology of the menstrual cycle has been studied in many different ways, from the point of view of neurobiologists, psychologists, and psychiatrists.

Possibly the most basic approach has been to study cyclic-dependent alterations in electroencephalogram (EEG). Early studies showed a slowing effect on the EEG during the premenstrual period. This has been confirmed recently by Wuttke, Arnold, Becker, Creutzfeldt, Langenstein & Tirsch (1975) who found frequency changes in the α-band but not in the bands which usually indicate abnormality.

Physiologists have studied changes in the autonomic nervous system (ANS).

Activation of the ANS is traditionally associated with states of tension and anxiety. Research in this field has concentrated on changes in heart rate, respiration rate, skin potential and sweat gland activity.

The only consistent changes found are those which reflect the luteal rise in body temperature, such as a rise in heart and respiration rate (Little & Zahn, 1974). These authors also found a greater degree of autonomic activity at ovulation, coinciding with a greater feeling of elation and vigour in their subjects.

A third approach has been to study psychomotor tasks and work performance in the cycle, with a view to social and economic aspects. Dalton (1960) reported a decrease in work performance in 27% of school-girls premenstrually, although 17% also showed improvement. Pierson & Lockhart (1963) studied simple reaction time and speed of arm movement and could find no differences at various stages of the cycle. Redgrove (1971) looked at work performance of punch card operators and typists and she could find no differences in either group apart from a slight increase in output by two out of three typists in the late premenstruum.

Another group of studies have described cyclical correlations, that is the relationship between the phase of the cycle and the occurrence of specific events. Correlations have bben reported between premenstrual and menstrual phases and suicide or attempted suicide (Ribeiro, 1962;Mandell & Mandell, 1967), admission to a hospital with acute psychiatric illness(Janowsky, Gornay, Castelnuovo-Tedesco & Stone, 1969) and accidents (MacKinnon & MacKinnon, 1956). But it has been pointed out by Parlee (1973) that some cyclical correlations are citations of early work which is anecdotal. For instances an anecdotal report of three crashes by menstruating women airline pilots (Whitehead, 1934) is often cited as an example of a cyclical correlation (Moos, 1968:Dalton, 1964).

In summary, the work on autonomic nervous activity and work performance in the cycle is rather incomplete in some cases and the relevance to the study of mood and behaviour is unknown. Similarly the cyclical correlations present problems in interpretation, since it is difficult to know how far they can be generalised to all women. However by far the largest group of publications have studied mood disorder, and these are described in the next section.

2. THE PREMENSTRUAL SYNDROME

The first report in the medical literature was by Frank (1931) who noted "rare and unpleasant" symptoms in the premenstrual phase of the cycle. One or two striking cases were noted in the literature, such as premenstrual weight gain of 12 to 14 pounds (Thomas, 1933). The first indication that premenstrual symptoms might be more common came from Stieglitz & Kimble (1949) and Morton (1950). Stieglitz & Kimble studied 67 women with complaints of some premenstrual discomfort and found that a majority suffered from emotional instability, headache, and breast engorgement, while half complained of backache and abdominal swelling. Morton (1950) looked at 29 patients and found that all had "emotional instability", and he also mentioned increased appetite and lower abdominal pain as important symptoms. Oedema of the face, hands and ankles was first noted by Frank (1931) and Thomas (1933) and later by Morton (1950).

At about this time two studies had been carried out which looked looked at cyclical changes in small groups of women not selected for premenstrual symptoms. Benedek & Rubenstein (1939) used an approach by both psychologist and gynaecologist to study the menstrual cycle. Altmann, Knowles & Bull (1941) studied ten women in detail and found a premenstrual outburst of physical and mental activity coupled with high tension, irritability and depression.

2.1. Terminology

R.T. Frank, the first author to describe the condition in 1931, first used the term premenstrual tension. He described a premenstrual feeling of "indescribable tension" with irritability, and " a desire to find relief by foolish and ill-considered actions".

This name has been criticised several times since then.

Greenhill & Freed (1940) suggested the term "toxaemia of menstruation" on account of the clinical similarity to pre-eclamptic toxaemia of pregnancy, which has early symptoms of depression, irritability, lethargy, headache, and nausea (Dalton, 1977).

In Britain the term premenstrual syndrome was introduced by Greene & Dalton (1953), and was widely adopted. However, pre-menstrual tension was still considered the most descriptive name by Lloyd (1963) since it was considered to reflect the state of the tissues as well as the emotions.

A further suggestion was put forward by Sutherland & Stewart (1965). They preferred the terms "cyclical syndrome" and "cyclical tension state",.with the former referring to the physiological syndrome occurring in most women, while the latter term describes the more severe form which might merit treatment. One reason for their preference of these terms is that the word "premenstrual" is omitted, since the complex of symptoms sometimes occurs in the absence of menstruation for instance at the climacteric (Greene & Dalton, 1953).

The terms in common usage are either premenstrual tension or the premenstrual syndrome (PMS), and this last term will be used in the following pages.

2.2 Symptomatology

The chief symptoms experienced by PMS sufferers have been described by several authors and the frequency of the main symptoms in these patients are summarised in Table 1.1. The differences in frequency of the symptoms in Table 1.1 are probably indicative of differences in the terminology of the various authors, since two groups of symptoms emerge from all the papers notably emotional

Authors / Patients	Stieglitz & Kimble (1949)	Morton (1950)	Greene & Dalton (1953)	Rees (1953)	Kerr(1977) * Taylor(1977)
Percentage with:					
Depression	(-)	(-)	60	80	64
Irritability	68	(-)	69	100	64
Lethargy	(-)	(-)	13	63	27
'Emotional lability'	(-)	100	(-)	(-)	(-)
Breast tenderness	65	(-)	24	63	27
Oedema	(-)	45	(-)	73	36
Headache	50	31	69	63	22
Backache	47	(-)	(-)	(-)	(-)
Increased appetite	(-)	59	(-)	(-)	(-)
Nausea	(-)	30	(-)	37	(-)
No. patients	67	29	84	30	120*

* combined figures. (-) indicates that the symptom or term was not mentioned by the author.

Table 1.1. Symptoms commonly reported in the literature for the premenstrual syndrome.

symptoms and feelings of oedema and breast discomfort. Psychological symptoms, such as depression, irritability, lethargy and confusion, are undoubtedly the most important, although bloatedness of breasts and abdomen, and headaches, frequently occur.

However many other symptoms have been described in the premenstrual phase, and Moos (1969) has found references to as many as 150 symptoms in the literature. Dalton (1977) has classified the wide variety of symptoms which may occur cyclically. There are psychological, neurological, respiratory, and dermatological symptoms, as well as orthopaedic disorders such as joint pains. Earlier workers had reported such symptoms as weight gain (Thomas, 1933) ulcerative stomatitis (Israel, 1938), increased thirst, insomnia, and nausea (Rees, 1953). In addition existing illnesses such as asthma, glaucoma, or epilepsy may be exacerbated cyclically (Dalton, 1964).

The timing of the symptoms in relation to menstruation is an important question which can only be resolved properly by the use of prospective studies. In one such study Moos, Kopell, Melges, Yalom, Lunde, Clayton & Hamburg (1969) found that symptoms related to swelling or weight gain began to occur at 14 days before the period, reached a peak and stayed high for 4 days premenstrually. Feelings of anxiety and aggression also increased after ovulation, with aggression reaching a peak 9 days premenstrually while anxiety was highest one to two days before menstruation. It is interesting to note that they found no separate peak at ovulation for any of these symptoms in their subjects, who were women selected for both high and low premenstrual symptoms.

Using retrospective questionnaires, other authors have usually stated that the symptoms last from seven to ten days and are usually,

but not always, relieved at menstruation (Rees, 1953; Greene & Dalton, 1953).

2.3 Definition

Studies of symptomatology have led several authors to try to define the syndrome. But faced with such a multiplicity of symptoms, Dalton (1977) has stated that the only common factor is the cyclical recurrence of disease with each menstrual cycle. The same conclusion was reached by Sutherland & Stewart (1965). Perhaps a more useful working definition was provided by Kessel & Coppen (1963) (see below), using epidemiological methods. They found a cluster of symptoms which were depression, irritability, tension, swelling and headache, which were maximal one to two days before menstruation. The feelings of swelling occurred in the breast (80%), abdomen (50%), feet and ankles (10%), and areas such as face, neck and fingers in a negligible number of women.

These symptoms closely resemble the clinical observations of Rees (1953) and Greene and Dalton (1953). In a recent study in this Department (Kerr, 1977; Taylor, 1977) of 120 women the syndrome was characterised by mental symptoms (70%) usually of depression or irritability or both while bloatedness, breast tenderness, headaches and lack of coordination occurred in 20 to 30% of patients (see Table 1.1).

2.4 Epidemiology

The premenstrual syndrome has been studied quite extensively since the 1950's. The studies have fallen into groups, where either whole populations or selected groups of patients have been studied. The former studies have helped to answer questions about the prevalence of the syndrome amongst all women. An early estimate was supplied by Bickers & Woods (1951) who found that 36% of all women in one

American factory applied for treatment in the premenstrual phase.

In a well controlled and now classic study Kessel & Coppen (1963) set out to look at the prevalence of premenstrual symptoms in British women sampled from general practices throughout Britain. 465 women were asked in retrospect about their symptoms by postal questionnaire. It was found that one in nine women reported severe pain, irritability or headaches before or during menstruation, while four out of five reported some swelling. A cluster of symptoms was found, of irritability, depression, tension, headache, and body swelling, all of which were maximal one or more days before menstruation, and this cluster occurred to a moderate or severe extent in one in four women.

These authors also found that dysmenorrhoea were present in 45% of all women but that those with the cluster of premenstrual symptoms developed pain before rather than during the menses. Thus one form of period pain is present with PMS, while true dysmenorrhoea occurs separately.

Sutherland & Stewart (1965) studied 150 students and nurses by retrospective questionnaire and they found that 66% experienced some dysmenorrhoea and 70% experienced cyclical skin eruptions. These high figures may be due to the young age group of their subjects (mean age 21). They also found that about 65% of the women experienced depression, irritability and body swelling premenstrually. These three symptoms occurred together in 39% of their sample.

Estimates of the prevalence of the premenstrual syndrome, as opposed to individual symptoms, varies widely in the literature due to the difficulties of definition of the syndrome.

Although figures as high as 95% have been quoted (Pennington, 1957) there is general agreement that the cluster of emotional and physical symptoms usually defined as PMS occur to a marked degree in

30 to 40% of women (Kessel & Coppen, 1963; Dalton, 1977).

The epidemiological approach is used in the study of the aetiology of many diseases, and may be helpful in the understanding of PMS. Dalton (1977) has noted that the syndrome tends to occur more frequently in women in their thirties and forties. She also notes that PMS is associated with high parity, indicating perhaps that childbirth is a precipitating factor, although parity is also related to age, so that it is difficult to state with certainty that it is associated with PMS. Another observation by Dalton (1964) is that PMS may persist after hysterectomy, ovariectomy, adrenalectomy or the menopause. However very few authors have studied these factors quantitatively. In one prospective study (Beumont, Richards & Gelder, 1975) it was found that premenstrual symptoms persisted after hysterectomy, but occurred to a lesser extent.

A large amount of work has been carried out on the effects of oral contraceptives on PMS. While there is some evidence that women experience less PMS while on the pill (Royal College General Practitioners, 1974) it also appears that PMS may be more frequent in past users of the pill (Kutner & Brown, 1972). There also appears to be an increased incidence of PMS after pre-eclamptic toxaemia (Dalton, 1977).

3. THEORIES REGARDING THE AETIOLOGY OF THE PREMENSTRUAL SYNDROME

Frank (1931) postulated that a rise in "female sex hormone" (oestrogen) might be responsible for premenstrual tension. He irradiated the ovaries of his patients and the symptoms were relieved. Since that time many treatments have been employed, including progesterone, progestogens, diuretics, androgens, vitamins, lithium and tranquillizers. Some of these treatments have been used empirically and success has been claimed in most cases, but in the few controlled studies carried out a very high placebo effect has been noted (Jordheim, 1972; Mattsson & Van Schoultz, 1974). Therefore apart from some very recent studies on bromocryptine, studies of treatments have not been very informative and they will not be considered in detail in the following review.

Before any connection can be made between any hormones and PMS, it must be established firstly, that specific biochemical changes may lead to behavioural change, and secondly, that these biochemical changes may be brought about directly or indirectly by one of the hormonal changes in the cycle.

A great deal cf attention has been paid to the monoamines and the effect of various drugs on peripheral nerve function. Unfortunately less is known about the central nervous system since by its very nature direct measurement is difficult.

There is indirect evidence that the central monoamines may be deficient in depression. The drug reserpine, which depletes the brain of monoamines, causes depression and another group of drugs, the monoamine oxidase inhibitors, prevent the degradation of the central monoamines, and these are sometimes successful in the treatment of depression (Pare & Sandler, 1959). The study of cerebrospinal fluid

(CSF) is the closest yet reached to the study of changes in the human brain and it has been demonstrated that depressives have lower CSF concentrations of indoleamines such as 5-hydroxytryptamine (5-HT) than non-depressed patients (Ashcroft, Crawford, Eccleston, Sharman, MacDougall, Stanton & Binns, 1966). Although it is still believed that amines are involved in depression, more recent work indicates that the relationship between depression and biogenic amines in the CSF is not straightforward (Pullar, 1973) and it is postulated that other factors such as receptor sensitivity are involved (Shaw, Riley, Tidmarsh & Blazek, 1976 ; Green, 1978).

It is an attractive idea to relate the changes in PMS to changes in the central amines, although there is little evidence to support this. Grant & Pryse-Davies (1968) found that the cyclic pattern of MAO in the endometrium is abolished in women taking the combined progestogen-oestrogen contraceptive pill and these pills are also responsible for a reduction in mood swings.

This is not of course evidence of a causative relationship. In a recent study Feine, Belmaker, Rimon & Ebstein (1977) looked at platelet MAO in women with PMS. They could find no cyclical change in a PMS group but in an earlier paper (Belmaker et al 1974) these workers found a marked cyclical pattern in a group of asymptomatic women. However the two papers are not strictly comparable.

Despite this lack of understanding of the central effects in PMS, several theories of aetiology have been put forward and these are considered below.

3.1. The possible role of ovarian steroids

Since the first suggestion by Frank (1931) that oestrogens might be a factor in PMS, attention has focussed on the ovarian steroids, especially the two most important steroids of the corpus luteum,

progesterone and oestradiol. Authors in early papers studied these hormones using indirect clinical means. Israel (1938) thought that PMS was caused not by an excess of circulating oestrogen but by a lack of antagonism by progesterone. He found that a majority of his patients had an abnormal secretory phase as indicated by endometrial biopsy, indicating a relative deficiency of progesterone, although he could not understand why PMS was not seen in anovular cycles.

Morton (1950) found a monophasic temperature curve in 23 of his 29 PMS patients and endometrial biopsy and vaginal smears suggested an excess of oestrogen to progesterone, with evidence of diminished luteal activity. He also gave oestrogens to two ovariectomised and one postmenopausal woman, who then suffered painful breasts, abdominal bloating, weight gain and "nervous tension". The general belief that the ratio of progesterone to oestrogen is low in PMS has been widely supported (Rees, 1953; Greene & Dalton, 1953). The support of the last two groups for this idea came largely from the success in treating PMS with progesterone injections or suppositories, although the placebo effect was not determined. Rees (1953) did however compare the effects of diuretic, progesterone, progestogen and androgen, and he found that progesterone, progestogen, and androgen were effective in alleviating physical and mental symptoms. He explained these results in terms of antagonism towards oestrogens, since both the progestogen (ethisterone) and the androgen (testosterone) are diuretic and antagonistic to oestrogens. He thought that oestrogens might cause PMS symptoms such as breast changes, water retention, and possibly hypoglycaemia and increased glucose tolerance.

More direct evidence regarding the role of the ovarian steroids has come in recent years from studies of hormone plasma concentrations. Loraine & Bell (1971), could find no abnormality in

the excretion of pregnanediol by six women with PMS.

Adamoupoulos, Loraine, Lunn, Coppen & Daly (1972) also looked at urinary steroid levels in a group of 15 women with PMS. They found that seven out of fifteen cycles were apparently anovulatory, as pregnanediol levels were very low in the second half of the cycle. However urinary gonadotrophins and oestrogens were not markedly altered. These authors concluded that, as the symptoms were still present in these anovulatory cycles, progesterone per se was not the cause of PMS, and neither was the renin-angiotensin-aldosterone system likely to be involved as this does not have a cyclical pattern in anovulatory cycles. These results seem rather strange in that later studies of plasma steroids, described below, have not recorded anovulatory cycles. Also some plasma studies have recorded raised oestrogen levels in PMS (see below). However in another urinary study (Prill & Krüger, 1963) no difference was found in urinary oestrogen levels in women with PMS. It could be that the plasma oestrogen differences are so small that they are not likely to be detectable in urinary excretion rates, since the latter reflect clearance rates as well as secretion. A more sophisticated approach was taken by Bäckström & Carstensen (1974), who measured hormones in blood.

These authors compared daily plasma levels of steroids in the last six days of the cycle from eight women with no symptoms and ten women with premenstrual symptoms of anxiety and irritability. They found that progesterone levels at one to three days before menstruation were the same in both groups but at 4-6 days premenstrually levels were significantly lower in the PMS group. Oestrogen levels were significantly higher in the PMS group in the two to five days before menstruation, resulting in a higher oestrogen/progesterone ratio at days 3 to 6 premenstrually.

In a further study Bäckström, Wide, Södergård & Carstensen (1976) looked at progesterone, oestrogens, FSH, LH and sex-hormone-binding globulin in the last ten days of the cycle and they found that at days 4 to 10 premenstrually both progesterone and oestrogens were low in a small group of women with PMS. However the oestrogen values rose to a peak above controls 4 days premenstrually while progesterone continued to be low. There were no differences in LH values or in levels of sex-hormone-binding globulin in the two groups. But FSH levels were higher in the PMS group 6 to 9 days premenstrually, then falling to control levels. Two other brief reports have appeared regarding ovarian steroids and PMS, although no details are supplied about methods. Smith (1975), in a study of premenstrual depression, found lowered progesterone levels, but no difference in plasma oestrogens. The one contradictory finding, by O'Brien & Symonds (1977) was that the progesterone peak was raised in women with PMS compared to controls.

It appears that the type of symptom is important, since further work by Bäckströms group on very small numbers has indicated no abnormality in sex steroid ratios of women suffering with either headaches (Bäckström & Carstensen, 1974) or feelings of swellings (Bäckström & Mattsson, 1975) as their main symptom. There is also evidence that women with benign breast disease may have reduced progesterone to oestrogen ratios (Sitruk-Ware, Sterkers, Mowszowicz & Mauvais-Jarvis, 1977). Unfortunately these authors do not indicate whether the women experience any other symptoms cyclically, indeed, the pattern of occurrence of breast disease is not recorded.

Thus the classification of premenstrual symptoms is an important factor which may confuse the endocrine picture, For instance

migraine is often cited as a premenstrual symptom (Dalton, 1964), yet in a study of 138 women with migraine only 24 had migraine attacks which could be related to the cycle, or menstruation (Epstein, Hockaday & Hockaday, 1975). Oestradiol and progesterone plasma levels were studied in eight of this "cyclical migraine" group compared to controls, and both hormones were found to be higher in women with cyclical migraine. It appears that migraine, although it may occur premenstrually or menstrually, cannot be considered as a PMS symptom but rather as a related but separate disorder.

The theory that gonadal steroids influence behaviour has a general grounding in the study of the role of these hormones in brain function in other mammals. There is now considerable evidence that oestradiol and progesterone accumulate in the central nervous system, and that they affect brain metabolism and neural function, and this is reviewed by Bäckström (1978).

For instance, autoradiographic studies show that selective accumulation of radioactive oestradiol occurs in the pituitary, hypothalamus, pre-optic area and brain stem of rats, cats and monkeys (Michael, 1965).

The cortex, subcortex, brainstem and cerebellum in the rat all take up radioactivity very quickly if ^{3}H-progesterone is injected, but the radioactivity is lost much more quickly from these areas than from the hypothalamus (Hamburg, 1966). This is not to say that progesterone has no functional significance in these areas, since the same phenomenon occurs for the potent anaesthetic pregnanolone, which still exerts anaesthesia 30 minutes after 98% of the steroid has disappeared from the brain in mice (Holzbauer, 1976).

There is also evidence from animals that progesterone and

oestradiol affect neural activity (Barraclough & Cross, 1963; Sawyer & Kawakami, 1961) and that they have antagonistic effects on electroshock seizures threshold in rats (Wooley & Timiras, 1962). Gonadal steroids also influence mating behaviour in rodents - and such behaviour correlates with the menstrual cycle in primates (Goy & Resko, 1972).

However there has been very little progress in the study of relationships between steroids and the human brain, due to the practical and ethical problems of such studies. It has been shown that total levels of progesterone, oestradiol and testosterone in human plasma are closely correlated with levels in cerebrospinal fluid, indicating that these steroids penetrate the central nervous system (Bäckström, Carstensen & Södergård, 1976a).

Epileptic women exhibit a cyclical pattern in the number of seizures, with least in the luteal phase, and an increase in the number at menstruation when progesterone is low (Laidlaw, 1956). Exogenous progesterone reduces the number of seizures and oestrogens increase them (Morrell, 1959). It has been known for a long time that progesterone is also a potent anaesthetic, and this effect is more marked in females (Merryman, 1954). But apart from the early psychoanalytical studies of Benedek & Rubenstein, (1939) correlating behaviour with vaginal cytology, there has been no direct study of human behaviour correlated with the ovarian cycle. Even in a very recent, and otherwise very thorough study, a peak in human female sexuality was found at midcycle, but simultaneous hormone measurements were not carried out to determine the exact relationship (Adams, Ross Gold & Burt, 1978).

3.2 The renin-angiotensin-aldosterone system

Several investigators have put forward the theory that an imbalance of electrolytes may be responsible for the symptoms of PMS, perhaps in conjunction with ovarian steroid imbalance. Greenhill & Freed (1941) first suggested that ovarian activity may influence electrolyte and water balance. Janowsky, Berens & Davis (1973) elaborated upon this theory, and put forward the hypothesis that the renin-angiotensin-aldosterone(RAA)system is involved.

They studied eleven women on a metabolic ward on a controlled diet over 15 cycles and they made daily evaluations of weight, 'negative mood', and the urinary potassium/sodium ratio (K/Na). They found a correlation between weight gain, negative mood and the urinary potassium/sodium balance, all of which reached a peak in the last seven days of the cycle. They also noted that the peak in K/Na ratio occurred two days before the peaks in weight gain and negative mood, suggesting a definite time course of events. It is known that the K/Na ratio in urine may reflect changes in the circulating levels of aldosterone (Morris & Davis, 1974). From their indirect evidence Janowsky et al (1973) infer that the RAA system may be involved in PMS.

The findings of Janowsky et al (1973) differ from previous reports in that they found a relatively small midcycle weight gain. In a similar controlled study Bruce & Russell (1962) found a greater and more abrupt change in weight at midcycle than in the premenstruum. but they did find a correlation between change in weight and sodium and water retention in their PMS patients.

The pattern of aldosterone excretion and also plasma levels occur in parallel with weight gain, with a peak at ovulation and again in the luteal phase (see page 22).

Perrini & Piliego (1959) found that the premenstrual excretion of aldosterone was increased up to threefold the post-menstrual in seven women with premenstrual syndrome. They also found a greatly increased K/Na ratio in urine in the premenstrual compared to the post-menstrual phases in their PMS patients. The change in K/Na ratio and aldosterone excretion was not seen in three non-PMS controls, who tended to have lower excretion of aldosterone and K/Na ratios at both times in the cycle. These findings regarding aldosterone excretion were confirmed by Piliego, Rossini, del Zotti & Scardapane(1964) who studied 15 patients and 15 controls.

There is apparently no other direct evidence to implicate aldosterone, although Greenhill & Freed (1941) found that diuretics relieved symptoms in 34 out of 40 patients. Rees (1953) found that diuretics relieved bloatedness but not depression.

Possible mechanisms causing symptoms of PMS

Changes in the RAA system and hence electrolyte balance is the obvious mechanism for the bloatedness and oedema seen in PMS. Yet even this relationship is in doubt. Oelkers, Narsen, Molzahn, Lohmann & Hammerstein (1973) studied sodium, oestrogen, aldosterone and potassium excretion in 3 patients with premenstrual weight gain of more than 3 Kg. They found that sodium was retained, yet aldosterone, oestrogen and renin excretion tended to be low. A salt-retaining factor other than aldosterone (or oestrogen) was postulated.

The emotional and psychological changes in PMS must also be explained. There are two possible mechanisms whereby symptoms may be produced. The symptoms may be caused indirectly via the retention of sodium or water brought about by increased aldosterone, and the retention of sodium or water may directly affect brain function. There is some evidence that the distribution of sodium and water are altered

in chronically depressed patients (Coppen & Shaw, 1963), and the distribution returns to normal when the patients recover.

The other possibility is that changes in aldosterone metabolism directly affect brain function. There is no evidence for effects of aldosterone on brain biochemistry or function, but angiotensin appears to alter animal behaviour, central neurotransmitters and autonomic function (Janowsky et al., 1973).

In summary, there is little direct evidence for the involvement of the RAA pathway. But as already described (p.24) progesterone may be directly responsible for the luteal increase in plasma aldosterone, yet progesterone itself has an antialdosterone effect. Oestrogens, which promote salt and water retention as described previously, may also alter the RAA axis. Therefore it is possible to imagine that an altered progesterone/aldosterone ratio may exaggerate the effect of some component of the RAA system, leading to at least some of the symptoms of PMS.

3.3 Prolactin

Horrobin (1973) has put forward a case for considering prolactin as a key factor in the syndrome. He proposed that prolactin causes both the mental and physical changes in the cycle, and that these two may be independent of each other so that a woman may have the mental symptoms but not the physical and vice versa. He also suggested that the syndrome is most likely to occur in women with an erratic pattern of prolactin secretion in the luteal phase of the cycle.

Some of the evidence is provided by Horrobin himself. He carried out a study of electrolyte balance in one PMS patient and he found that water and both sodium and potassium were retained in the last 7 days of the cycle. Prolactin, unlike the mineralocorticoids, retains water and both sodium and potassium. Retention of potassium in

the luteal phase has also been shown by Edwards & Bayliss (1973) in women in the upright position, although Bruce & Russell (1962) could find no change in potassium balance in PMS patients.

More direct evidence for the prolactin theory has come from the measurement of prolactin in serum. This was done on 4 occasions in the last three weeks of the cycle in 28 women with PMS and 21 controls (Halbreich, Ben-David, Assael, & Bornstein, 1976). They found that throughout the menstrual cycle the mean serum prolactin in the PMS group was higher than the controls. They also found that the average individual increase in the prolactin in the premenstrual period was higher in women with PMS. Despite great variability among the subjects, these differences were statistically significant.

Halbreich et al (1976) may thus shed light on the controversy over whether a premenstrual rise in prolactin occurs (see section 1.1.3) by the fact that previous samples of women were not classified by their PMS status.

Further information about the role of prolactin in PMS has been obtained from the study of the effects of bromocryptine, which suppresses prolactin secretion by a direct action on the pituitary (Pasteels et al,1971). Bromocryptine acts as an analogue to dopamine, which is the prolactin inhibitory factor, so that bromocryptine is a dopamine receptor-agonist. In four studies bromocryptine has been administered to PMS patients in a double-blind crossover fashion. In the first study (Benedek-Jaszmann & Hearn-Sturtevant,1976) the subjects were perhaps atypical in that they were patients attending an infertility clinic and had prolactin levels just above or below the upper normal limit. There was significant improvement in breast symptoms, oedema, weight gain and mood, and the reduction of prolactin levels, by bromocryptine.

Unfortunately these authors did not extend their double-blind trial to their other group of women who were not selected for infertility or high prolactin values. This group of women did however report good results after bromocryptine treatment on an empirical basis.

Other controlled trials have been less encouraging. Ghose & Coppen (1977) in a study of thirteen patients, found no significant improvement. In a more detailed study Andersen, Larsen, Steenstrup, Svendstrup & Nielsen (1977) found no improvement in any symptoms except 'mastodynia' in the bromocryptine-treated cycles. Of the 21 women in their study only five had transient high levels of prolactin and there was no evidence for a difference in follicular and luteal levels, in contrast to Halbreich et al (1976). However Andersch, Hahn, Wendestam, Öhman & Abrahamsson (1978b) in a study of 20 PMS patients and 20 controls, did find a luteal increase in prolactin in the PMS group, although only two samples were taken per cycle. These authors also reported that the women, by self-rating, were significantly improved in the symptoms of breast discomfort and irritability. The problem with comparing these studies is that different dosages and time schedules were employed, and different methods were used to evaluate improvement. Another problem is that bromocryptine, as dopamine agonist, may be acting on central mechanisms other than prolactin secretion.

The interaction between prolactin and the steroids of the menstrual cycle (p.26) may be implicated in the aetiology of PMS. While frank hyperprolactinaemia leads to anovulation, slightly raised prolactin levels may be responsible for menstrual cycle defects such as the short luteal phase (Corenblum, Pairaudeau & Schewchuk 1976). Benedek-Jaszmann & Hearn-Sturtevant (1976) have suggested a connection between prolactin-induced progesterone deficiency and PMS, since they found evidence that many of their infertility

patients with "high-borderline" prolactin also had short luteal phases and evidence of progesterone deficiency as shown by endometrial biopsy. All of their patients had premenstrual syndrome, yet it is not clear whether the sample was in fact selected for this criterion. Therefore all that can be said is that the premenstrual syndrome may occur in conjunction with slightly raised prolactin and luteal deficiency, and, indeed, Andersen et al (1977) observed three cases of slight hyperprolactinaemia and progesterone deficiency, of which only one had a typical PMS.

If prolactin is involved in PMS then some explanation of the symptoms must be made. Prolactin is known to promote mineral and water retention, which may explain the oedema seen. Moreover prolactin may be directly responsible for breast changes, and this would accord with the results of Andersen et al (1977) and Andersch et al (1978b) It is worth pointing out however that breast disease, such as galactorrhoea, may occur in spite of prolactin levels in the normal range (Kleinberg, Noel & Frantz, 1977), and also that many patients with hyperprolactinaemia do not have galactorrhoea (SeppÄlÄ, Lehtovirta & Ranta, 1977).

The possible role of prolactin in mental illness is reviewed by Horrobin, Mtabaji, Karmali, Manku & Nassar (1976). The interest of these workers was first aroused by the fact that some drugs such as reserpine which provoke depression also stimulate prolactin secretion. But the only published report of prolactin levels in depressive illness is that of Sachar, Frantz, Altman & Sassin (1973) who report that baseline prolactin levels were elevated in their 24 patients.

A preliminary report by Horrobin et al (1976) confirms that prolactin is raised above upper limits of normal in twelve patients with neurotic depression. These authors point out however that

emotional stress may give rise to high prolactin secretion and not vice versa.

It seems therefore that prolactin is unlikely to be the primary cause of neuronal disturbance, leading to deranged behaviour, especially as prolactin is within limits for controls in many patients (Andersen et al 1977, Andersch et al., 1978b). Perhaps prolactin reflects other changes in the neuroendocrine pathways which may be responsible for mood changes.

3.4 Other hormonal theories of PMS

Billig & Spaulding (1947) suggested that women with PMS have a mild degree of hyperinsulinism in the premenstrual phase, leading to hypoglycaemia. They carried out glucose tolerance tests and found that there was increased sugar tolerance immediately prior to menses compared to other stages of the cycle, and this was also found by Morton (1950). The symptoms of hypoglycaemia are hunger, increased peripheral nerve sensitivity, and irritability as well as possible headaches and loss of concentration. Easy bruising may also be part of this syndrome. These very early papers have not been followed up, although Freedman, Ramcharan, Hoag & Goldfien, (1974) in a study of over 4000 women, found that serum glucose was reduced in the last five to eight days of the cycle.

Dalton (1977) has put forward a general concept for the aetiology of PMS involving a fault in a "progesterone feedback pathway". She postulates a feedback pathway via the hypothalamus and pituitary to the adrenal cortex, which when interrupted would lead to diminished stimulation of the adrenal cortex and disturbances in glucocorticoids and mineralocorticoids.

Unfortunately there is no evidence for such a pathway. Progesterone has a synergistic effect on the negative feedback of

oestradiol on FSH via the hypothalamus, but no effect on the adrenal cortex has been reported. Progesterone does alter glucose in that it promotes amino acid breakdown by the liver (Landau & Lugibihl 1961), but this action is independent of the adrenal cortex since it is maintained in hypophysectomised, adrenalectomised patients (Landau, Bergenstal, Lugibihl, Dimick & Rashid, 1957).

Similarly the natriuretic effect of progesterone, as discussed in section 1.1.2. is also independent of the adrenal since the antialdosterone action of progesterone can be reproduced in adrenalectomised patients given aldosterone substitution therapy (Landau & Lugibihl, 1961). Thus the mechanism described by Dalton (1977) seems very unlikely.

There has however been no work on cortisol levels in PMS. There is evidence that a small increase in adrenocortical activity may take place in depressed patients but the disturbance is not as great as that seen in minor stress situations (see Coppen, 1970, for review).

A quite different theory was suggested by Rogers (1962). He pointed out that the symptoms of PMS such as irritability, depression, and oedema are present in many allergic conditions, and that many women may suffer from an allergy to the steroids themselves. Many allergies, due to small molecules such as drugs, involve the combination in vivo of the drug (acting as hapten) with a body constituent to form a complement antigen. One example is penicillin (Levine, 1965).

Heckel, Leahy & Pleger (1958) showed that many women with menstrual disorders were allergic to pregnanediol when skin tested with the substance, although none were allergic to oestrogens or progesterone. Rogers (1962) gave minute quantities (1-0.1 micrograms) of pregnanediol to 33 patients of whom 29 showed improvement. This in

itself is not remarkably but he claimed that the women could differentiate between doses of 0.1 and 1 micrograms with improvement at the lower dose, presumably with desensitisation, but worsening at the higher dose.

No further work has been reported with regard to the endocrine allergy theory of PMS, although it has been noted (Dalton, 1977) that many allergic conditions, such as asthma, rhinitis or urticaria, are exacerbated premenstrually.

Another substance implicated in the aetiology of PMS is pyridoxine (vitamin B_6). A number of workers originally speculated that the depression sometimes associated with oral contraceptives might be related to a functional deficiency of this vitamin (Rose, 1969) and pyridoxine has been used successfully in this type of depression (Adams, Rose, Folkard, Wynn, Seed & Strong, 1973).

Pyridoxine is a co-factor in the dopaminergic pathway and also in several steps in the metabolic pathway of tryptophan. It has been suggested (Brush, 1977) that a relative deficiency of B_6 might act at several levels to induce the symptoms of PMS, through dopaminergic pathways and via disturbed tryptophan metabolism. The only evidence so far comes from the use of vitamin B_6 therapy in PMS. An early controlled trial had disappointing results (Stokes & Mendels, 1972), although empirical treatment of 70 women has proved successful in over half the cases (Kerr, 1977).

3.5. The psychogenic theory of PMS

Although it is commonly believed that the mood changes of the menstrual cycle have biochemical origins, there are undoubtedly social and psychological factors. Menstruation itself is still surrounded with superstition and taboo. It may be argued that the tension, irritability and depression seen premenstrually could be

socially mediated responses to the onset of menstrual flow. This theory can be tested by studying women taking oral contraceptives where menstruation itself still occurs yet without the normal endocrine events, although there are problems as other psychological factors come into play in these women. Paige (1971) found that mood swings were abolished in women taking combination and sequential oral contraceptives, confirming the work of Grant & Pryse-Davies (1968).

Using a different approach, Berry & McGuire (1972) related the womans perception of her sexual role (using a questionnaire) with her cyclical symptoms, and they found no evidence that the classic PMS symptoms were associated with psychosocial or psychosexual conflicts.

Beumont, Richards & Gelder (1975) studied premenstrual symptoms in 25 women with normal cycles and 7 hysterectomised women. The phase of the cycle was determined by menstrual dates in the menstruating women and by LH and progesterone values in the hysterectomised group. Using a prospective self-rating daily questionnaire they found that the menstruating group had more pronounced swings in mood than the hysterectomised group, suggesting that the level of symptoms is dependent on the womens awareness of the stage of the cycle. But one puzzling feature is that the mean scores of mood, physical and psychological symptoms are higher throughout the cycle in the hysterectomised group, a feature which is not commented upon by the authors. It is likely that women after hysterectomy are an atypical group and therefore not suitable as controls.

Another commonly held belief is that PMS is associated with psychological ill health. According to this idea all women experience physiological change, but the women who complain about these symptoms are neurotic. It has been reported that premenstrual

symptoms occur in up to 95% of women (Pennington, 1957). This implies that PMS is intrinsic and physiological, and indeed several theories have been put forward which suggest a selective advantage to PMS in the course of evolution (Rosseinsky & Hall 1974; Morriss & Keverne, 1974).

In a study of 500 randomly sampled women Coppen & Kessel (1963) found a positive correlation between neuroticism and severe premenstrual tension. However, as Clare (1977) points out, the symptoms on the scoring system for neuroticism heavily overlap the symptoms used to score PMS, so it is not surprising that an association was found.

Thus there is no conclusive evidence so far that PMS is psychogenic in origin.

3.6 Aims of Thesis

From the discussion above it would appear that several hormonal theories of the cause of PMS have been pursued but none have been tested in a convincing manner.

The hormones most often mentioned are progesterone, oestradiol, prolactin and aldosterone. It was felt that the absolute values of these hormones may be less important than the relationships between them. It was decided therefore to try to measure plasma progesterone, oestradiol and aldosterone and serum prolactin simultaneously during the cycle in women with PMS and compare the relationships found with those in asymptomatic women.

Ideally one would like to study hormone relationships at the local level of the tissue affected, such as brain or breast tissue, but these are obviously inaccessible in human subjects, and no animal models for PMS exist. Another biochemical approach would be to study monoamines and their metabolites, but the same problem is encountered in that only peripheral blood can be studied in the women. In addition,

the methodological problems of measuring substances such as dopamine are considerable.

Therefore it was felt that an overall picture of hormonal events during the cycle was a good starting point, especially as all the hormones have not been studied simultaneously in women with PMS before. In this thesis it was also hoped to be able to study hormonal patterns associated with different symptoms, and also to compare some psychological and endocrinological approaches. The endocrinological studies proposed above have only been possible in the last decade, thanks to advances in techniques of plasma steroid measurement. These advances are reviewed in the next section.

4. EARLY METHODS OF MEASUREMENT OF STEROIDS

4.1. PROGESTERONE

Early measurement of progestational activity involved bioassay, the end-point of which usually included either maintenance of pregnancy, or changes in uterine physiology, such as hypertrophy of the stromal nuclei in ovariectomised mice (Hooker & Forbes, 1947).

Physicochemical methods of progesterone quantitation were developed in the 1950s, and the first successful determination of progesterone in blood was the spectrophotometric method of Zander & Simmer (1954). Numerous extraction steps and a paper chromatographic step were required to give the necessary specificity. Modifications of this method were published by Short (1958), Sommerville & Deshpande (1958) and Oertel, Weiss & Eik-Nes (1959), but all these methods were only sensitive enough for pregnancy plasma.

At about this time patterns of progesterone in the body were investigated by the measurement of its main metabolite, pregnanediol, in urine. Using the colorimetric method of Klopper et al (1955) clinically useful information was obtained about the normal menstrual cycle (Klopper, 1957).

A further improvement in assay of blood levels came with the development of gas chromatographic (GLC) methods (Collins & Sommerville 1964), which gave slightly improved sensitivity. Other useful GLC methods were developed using flame ionization (Yannone, McComas & Goldfien, 1964), or electron capture (Van der Molen & Groen, 1965). Another chemical method was the double derivative isotopic method where a radioactively labelled reagent such as ^{35}S- thiosemicarbazide is allowed to react with progesterone (Riondel, Tait, Tait, Gut & Little, 1965). After extensive purification steps the specific activity of the product is measured by liquid scintillation counting, and thus the mass of steroid can be determined. There is automatic

correction for losses as ^{3}H-progesterone is added initially as internal standard. Although the recovery of steroid was low in this method, the sensitivity was higher than could be previously achieved.

However all these methods were laborious, due to the number of purification steps involved, and they were therefore unsuitable for routine clinical use.

Saturation analysis using the competitive protein binding technique of Murphy (1967) was first applied to progesterone by Neill, Johansson, Datta & Knobil (1967), using a dog corticosteroid binding globulin. However the binding protein was not specific enough to eliminate all chromatography steps, so that thin layer chromatography was necessary (Neill et al., 1967, Yoshimi & Lipsett, 1968).

However Johansson (1969) reported a competitive protein binding (CPB) assay using one extraction with a particular petroleum ether which selectively extracts progesterone, making chromatography unnecessary.

The first radioimmunoassay (RIA) reported (Abraham, Swerdloff, Tulchinsky & Odell, 1971) was employed using an antiserum to the 21-hemisuccinate of 11-desoxycortisol. This antiserum cross-reacted completely with 17-hydroxyprogesterone and 11-desoxycortisol, so that a column chromatography step was required. Furuyama & Nugent (1971) prepared an antiserum to progesterone-3-carboxymethyl-oxime-BSA, but they found that the results in plasma were doubled unless a column chromatography step was included.

Recent RIA methods have used an antiserum to 11α-OH progesterone hemisuccinate BSA which is more specific (Spieler, Webb, Saldarini & Coppola, 1972; Youssefnajadian, Florensa, Collins & Sommerville, 1972). This has enabled assays to be carried out without chromatography, and since then many methods have been published with procedural improvements, enabling the rapid, routine estimation of

progesterone in blood. Two recent modifications have been the use of ^{125}I-iodohistamine radioligands (Scarisbrick & Cameron, 1975) and enzyme-labelled progesterone (Joyce, Read & Fahmy, 1977) as the 'labels' to be 'counted'.

4.2 OESTRADIOL

The problem of assay of oestradiol are those associated with the measurement of any steroid, namely those of specificity and sensitivity. The oestrogens present more difficulties than most, however, due to the existence of several structurally similar oestrogens, and also due to the various forms in which oestrogens may exist, such as conjugates in urine and protein bound complexes in blood. The earliest methods of analysis were bioassays measuring total nucleated or cornuated epithelial cells in the vaginal smear of ovariectomised mice (Allen & Doisy, 1923). This and other bioassays did however suffer from the usual draw-backs of tediousness, lack of reproducibility, and nonspecificity.

Techniques using chemical determination were later developed to measure oestrogens in urine since the methods were not sensitive enough to measure blood levels. The detection of oestrogens was carried out by a colorimetric reaction such as the Kober reaction where the sample is heated with aqueous sulphuric acid and phenol, cooled and reheated to reveal an absorption maximum at 520 nm. This method was used by Brown (1955b) to measure urinary oestrogens and he was able to assay oestrone, oestradiol and oestriol individually using prior chromatographic steps.

The reaction was modified (Ittrich, 1958; Klopper & Wilson, 1962), but problems of sensitivity were still present, especially for blood samples. Other chemical methods such as acid fluorescence (Preedy & Aitken, 1961) and gas liquid chromatography (Wotiz, Charransol & Smith, 1967) have been used to measure the individual

oestrogens in non-pregnancy plasma, but large volumes of blood are required, precluding studies where repeated sampling is necessary.

When radioactive oestrogens became available these could be used as internal standards to determine losses during the lengthy purification procedures. They were also used in the double isotope derivative technique together with a reagent such as ^{35}S-p-iodobenzene sulfonyl chloride (Baird, 1968). These techniques were sensitive enough to measure oestradiol-17β in the plasma of men and non-pregnant women, but the procedure was still very laborious.

Saturation analysis was soon applied to oestradiol using at first uterine binding proteins from rabbit (Korenman, Perrin & McCallum, 1969) and sheep (Shutt, 1969). These binding proteins were not specific enough to discriminate between the oestrogens without prior chromatographic separations. The Competitive protein binding assay of Korenman *et al* (1969) which used celite columns did however show good agreement for male and female follicular phase values with the isotope dilution method of Baird (1968).

A recent step in the assay of plasma oestradiol has been the elimination of the chromatography step by using a highly specific antiserum. Abraham(1969) carried out the first non-chromatographic assay of plasma oestradiol but the antiserum was fairly nonspecific.

Other RIA methods described for oestradiol have had to include column chromatography but these assays were improved by the use of Sephadex LH-20 microcolumns (Emment, Collins & Sommerville, 1972) which speeded up the assays and reduced the assay blank effect.

Eventually antisera were produced with a cross-reactivity of less than 2% with oestrone and oestriol, which meant that non-chromatographic assays could be performed. These antisera were produced

against either oestradiol-11-BSA (England, Niswender & Midgley, 1974) or oestradiol-6-BSA (Dean, Exley & Johnson, 1971).

More recent assays have eliminated even the ether extraction step. Possible interfering substances are removed by adding an excess of testosterone to displace all oestradiol from plasma proteins (Jurjens, Pratt & Woldring, 1975). Other simplifications have been the use of iodinated labels for oestradiol for counting by gamma ray spectrometry (England et al., 1974), and even enzyme labels (Van Weeman & Schuurs, 1971).

4.3 ALDOSTERONE

The initial assays for mineralocorticoid activity were crude bioassays which measured changes in the level of sodium or potassium excretion by adrenalectomised dogs or rats. This method was modified by Simpson & Tait (1952) so that radioactive sodium and potassium were used.

After aldosterone itself was isolated and characterised a number of physicochemical methods were developed. In one such method the end-point depends on the ability of the aldosterone ketol side chain to reduce tetrazolium salts which then produce soda fluorescence (Nowaczynski, Koiw & Genest, 1957). However many other groups react with the tetrazolium salts so that successive chromatography steps were required first. The majority of the chemical methods were used to measure the urinary metabolite of aldosterone, aldosterone-18-glucuronide, which is converted to the free compound by hydrolysis.

The secretion rate of aldosterone could be measured by the adminstration of ^{14}C- or ^{3}H-isotopes and the estimation of the specific activity of the metabolite aldosterone-18-glucuronide in blood. Estimates of aldosterone secretion rates in clinical conditions have confirmed the trends seen in the urinary excretion of aldosterone (for review see Coghlan & Blair-West, 1967).

An isotopic dilution method of measurement which was more sensitive and therefore suitable for assay in plasma was described in the late 60's. The methods of Peterson (1964), Coghlan & Scoggins (1967) and Nowaczynski, Silah & Genest (1967) involve reacting plasma aldosterone with ^{14}C-acetate anhydride while ^{3}H-aldosterone is used as internal standard. These methods are sensitive since the sensitivity depends only on the specific activity of the labelled reagents, but the specificity relies upon several purification steps.

Another method, gas liquid chromatography, was also developed about this time (Bravo & Travis, 1967) but was only sensitive enough for urinary studies.

All these physico-chemical methods have the drawback of nonspecifity, which is especially important in the case of aldosterone, since this hormone is present in blood in very low concentrations together with large amounts of the other corticosteroids. The daily secretion rate of aldosterone by the adrenal is 50-100 µg while the corresponding rate for cortisol is 8-25 mg. (Catt, 1971). None of the above methods were therefore practical enough for routine clinical use, especially for plasma estimations.

More information became available after the application of competitive binding protein techniques. Unfortunately aldosterone is not bound to any extent by plasma proteins (Davidson, De Venuto & Westphal, 1962), so that intracellular binding proteins had to be used. However attempts to use renal receptor proteins were not successful owing to the instability of the proteins (Robinson & Fanestil, 1970; Vyzantiades, Ekins & Slater,1970).

The first reports of radioimmunoassays for plasma aldosterone still involved a chromatographic step (Mayes, Furuyama, Kem & Nugent, 1970; Bayard, Beitins, Kowarski & Migeon, 1970), since the antisera, to aldosterone-3-oxime BSA, were relatively nonspecific. An

antiserum to aldosterone-18, 21-dihemisuccinate BSA has been made by Haning and coworkers at the Worcester Foundation (Haning, McCracken, St. Cyr, Underwood, Williams & Abraham, 1972). This antiserum, although also rather nonspecific, has been widely distributed and has led to a large increase in the number of reports on aldosterone plasma levels.

More recently several highly specific antisera have been developed which eliminate the chromatographic step. This was achieved by the careful preparation of a pure mono-oxime conjugate, aldosterone - 3 - carboxymethoxime - BSA (McKenzie & Clements, 1974; Jowett, Smith & Slater, 1975; Vetter, Freedlender & Haber, 1974). On theoretical grounds conjugation at the C-3 position which exposes the 'Unique' C and D rings of aldosterone would produce more specific antibodies than conjugation at the C-18 or C-20 positions.

CHAPTER 2

Methods

1. GENERAL METHODS

1.1 Materials

Steroids

Cortisol(11β,17α,21-trihydroxy-4-pregnene-3,20-dione), oestrone (3-hydroxy-1,3,5 (10)-estratriene-17-one), and 17α-hydroxyprogesterone (17α-hydroxy-4-pregnene-3,20-dione) were from B.D.H. Ltd., Poole, Dorset.

Aldosterone(18 ,11-hemiacetal of 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al), oestriol(1,3,5 (10)-estratriene-3,16α,17β-triol), pregnenalone(3β-hydroxy-5 -pregnen-20-one), pregnanedione(5β-pregnan-3,20-dione), dehydroepiandrosterone(3β-hydroxy-5-androstene-17-one), 11α-hydroxyprogesterone(11α-hydroxy-4-pregnene-3,20-dione), 20α-hydroxyprogesterone(20α-hydroxy-4-pregnene-3,20-dione) and téstosterone(17β-hydroxy-4-androstene-3-one) were from Sigma Chemical Co. Ltd., Kingston, Surrey.

Cholesterol(4-cholestene-3β-ol), corticosterone(11β,21-dihydroxy-4-pregnene-3,20-dione), oestradiol-17β (1,3,5 (10)-estratriene-3,17β-diol), and progesterone(4-pregnene-3,20-dione) were from Koch-Light Laboratories,Ltd., Colnbrook, Bucks.

11-Deoxycorticosterone, DOC(21 hydroxy-4-pregnene-3,20-dione), tetrahydrocortisol(3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one), 18 -hydroxydeoxycorticosterone, 18-DOC(18,21-dihydroxy-4-pregnene-3,20-dione) and aldosterone, was from the MRC Reference Collection, Westfield College, London NW3.

DHD (20αhydroxy-9β,10α-pregna-4,6 -diene-3-one) and dydrogesterone (9β,10α-pregna-4, 6 -diene-3,20-dione) were from Philips Duphar,BV, Weesp, Holland.

Radioactive Steroids

(1,2-^{3}H) progesterone, 58 Ci/mmol, (1,2,6,7-^{3}H) progestérone, 87Ci/mmol, (1,2-^{3}H) aldosterone, 50Ci/mmol, (2,4,6,7-^{3}H) 17β-oestradiol, 85Ci/mmol,

(1,2,6,7-^{3}H) cortisol, 82 Ci/mmol, and ^{3}H-hexadecane standard were from the Radiochemical Centre, Amersham, Bucks.

Antisera

An antiserum to progesterone-11α-hemisuccinyl-BSA, raised in goats, was a gift to Dr. M. G. Brush from Dr. B. J. A. Furr, National Institute for Research in Dairying, Reading.

An antiserum(088) to aldosterone-18,21-dihemisuccinate-BSA, raised in sheep, was a gift to Dr. M. G. Brush from the National Institute of Arthritis, Metabolic & Digestive Diseases, Bethésda, U.S.A

Antisera to 17β-oestradiol-6(-o-carboxymethyl)-oxime-BSA, raised in rabbits, was given by Dr. P. G. Dean, Department of Biochemistry, University of Liverpool.

Progesterone 11α-hemisuccinate and progesterone-11α-hemisuccinyl-BSA were kindly provided by Dr. M. W. Johnson, Chelsea Hospital for Women, and the latter was used to raise antisera in our laboratory (see below).

General Reagents

Light petroleum(petroleum ether), bp 40°-60°C and bp 80°-100°C, ethyl acetate, toluene, chloroform, boric acid, sodium hydroxide, sodium dihydrogen orthophosphate($2H_2O$) and disodium hydrogen orthophosphate(anhydrous), and isatin, were all Analar grade, from B.D.H. Ltd.

1,4-diaminoanthroquinone, sodium azide, gelatin, Dextran Grade C mol wt. 60,000-90,000, and Decon 90 were also from B.D.H. Ltd.

Zimmermans reagent (2.5M potassium hydroxide in ethanol and 2% (w/v) m-dinitrobenzene in ethanol), prepared freshly before use, was also from B.D.H. Ltd.

Charcoal Norit A, Bovine Serum Albumin and Tris buffer, pH 8.5 were from Sigma Chemical Co. Ltd.

Other reagents were methanol, Analar(Hopkins & Williams, Chadwell

Heath,Essex), ethanol, Analar, (Burroughs &Wellcome, London S.E.11), dichloromethane, Pronalys (May & Baker, Dagenham, Essex), PPO, (2,5-diphenyloxazole) and dimethyl POPOP (1,4-(2-(4-methyl-5-phenoxazolyl))-benzene) from Koch-Light Laboratories, Ltd.

Sephadex LH-20 was from Pharmacia Fine Chemicals, Uppsala, Sweden.

Also used were Triton X-100(Rohm &Haas, Croydon, Surrey), Freunds adjuvant(Difco, Surrey) and butane/helium,1.3/98.7(B.O.C.,London S.W.19)

Glassware and pipettes

10, 15 and 30 ml capacity Quickfit tubes and stoppers were obtained from Corning Ltd., Stone, Staffs.

Volumes of 10 µl and 25 µl were dispensed using Eppendorf automatic pipettes (Eppendorf.GN, Hamburg, Germany) and volumes of 0.05, 0.1, 0.2 and 0.5 ml were dispensed using Oxford Samplers (Boehringer Corporation Ltd., London).

Volumes of 0.4 to 1.0 ml were also dispensed with an adjustable Pipetman 1000 (Gilson, Villiers-le-Bel, France) or a Selectapette (Clay Adams, N.J., U.S.A) which proved useful in deep narrow tubes.

For repeat dispensing of solvents, volumes of less than 1 ml were dispensed from an Oxford Pipettor (Boehringer Corpn. Ltd.)and above this volume a BB pipette was used (Bie & Bernsten,Ltd., Copenhagen) was used.

Volumes of scintillant were dispensed with a 10 ml Zippette (Jencons Hemel Hempstead, Herts.), and other scintillation equipment was glass vials (Packard Instruments Ltd., Caversham, Berks) and plastic insert vials (Sterilin, Teddington, Middx.)

3 ml capacity tubes for plasma storage were from Luckhams, Sussex.

Apparatus

Lithium heparin 10 ml tubes for blood samples (Teklab, Durham) were centrifuged in an MSE Super Minor. All other centrifugations were carried out in the MSE Mistral 4L (MSE, Crawley, Sussex).

A vacuum oven(size 1, Gallenkamp) was attached to a vacuum pump, 2SCDC (Edwards, Crawley, Sussex) for the evaporation of solvents in the assay tubes.

All other drying down procedures were using compressed air (on tap) and a manifold incorporated into a Perspex frame containing syringe needles which slotted on top of a Perspex rack to fit 12 x 100 mm tubes. This piece of equipment was made in the Medical Unit Workshop, St. Thomas's Hospital Medical School.

A Corumatic 200 liquid scintillation spectrometer, in use before October 1975, a 4π scanner (both from Tracerlab, Surrey), and an Intertechnique SL33 liquid scintillation spectrometer (Intertechnique, Brighton, Sussex), were used for radioactive counting.

A scanning spectrometer, Unicam SP800 was used in the Biochemistry Dept., St. Thomas's Hospital Medical School.

Tubes were vortexed using a Rotamixer de luxe (Hook & Tucker, London S.W.9) and for short periods they were kept at 4° C on a Cooltray(Chemlab Instruments Ltd., Hornchurch, Essex).

The calculators were a Compucorp 344 Statistician (Sumlock, California, U.S.A) and an HP55 (Hewlett Packard, Slough, Berks).

Other equipment was ; silica gel layer plates 60F254(Merck, Darmstadt, Germany), chromatography paper (Whatman, Maidstone, Kent), paper chromatography tanks (32 x 20 x 57 cm), thin layer chromatography tanks (22 x 7x21 cm) and a microlitre glass syringe to apply chromatographic samples (Hamilton Bonaduz AG, Switzerland).

Cleaning glassware

Glassware was rinsed with water several times before immersion in 5% Decon 90 for 8 - 24 hours. It was then rinsed in tap water at least 12 times and in distilled water 10 times, and dried at 75°C. Extraction tubes were prerinsed with 1-3 ml of the solvent to be used.

Plastic insert vials, Pasteur pipettes and automatic sampler tips were used once and discarded.

1.2.Scintillation counting

A quench curve was constructed for different volumes of buffer, using ^{3}H-hexadecane as internal standard. A regression analysis was performed for channels ratio (CR) and automatic external standards channels ratio (AESCR) curves using the Compucorp calculator. Regression analyses were linear ($r = .98$) for efficiences up to 30% on the Tracerlab. Regression equations for channels ratios in this range were used to calculate efficiences and hence dpm. For the Intertechnique coefficients from the quench curve were read into a computational unit via the Multimat Teletype of the SL33 before counting. A programme would then calculate a non-linear quench curve and automatically correct each vial so that both cpm and dpm were printed.

Triton X-100 detergent Triton X-100, a polyoxyethylene ether, is a detergent which has proved most useful in the counting of aqueous solutions, as it forms stable solutions with up to 14% water (Turner, 1971). 5 g PPO and 0.1 g dimethyl POPOP were dissolved, with stirring, in 667 ml. toluene, and 333 ml. Triton X-100 was added. Up to 15% of buffer could be mixed with the scintillant to form a clear solution.

The use of polythene insert counting vials

When large numbers of vials are in use, for instance,in radio-assay, the cost and practicability of the counting system must be considered. Polyethylene vials of 5 ml capacity which fit

into the standard glass vials may be of advantage for two reasons. Firstly, the vials are cheaper than the glass ones, so that they may be thrown away after use, which eliminates the problem of washing and decontamination. Secondly, less scintillant is needed, (and this may partly offset the increased cost of the disposable vials!).

It was noticed on the Intertechnique, if the same insert vials are recounted, that there was an increase in the computed dpm of approximately 130% over 15 hours if the AESCR quench curve was used. However if the CR quench curve was used changes with time did not occur, so that this method of quench correction was used in subsequent assays. Nevertheless, to counteract any possible changes with time, standard curve vials were placed both before and after unknowns symmetrically about an axis, thus: 1,3,5 .... 6,4,2.

The final counting system was 3.5 ml Triton mixture in polyethylene insert vials, and efficiency under these conditions, using 0.5 ml of 0.1 M Phosphate buffer as quencher, was about 19% on the Tracerlab (compared to an efficiency of 23% in all glass vials) and 26% on the Intertechnique.

1.3 Purification of reagents

1.3.1 Radioactive steroids

Although the half-life of ^{3}H is twelve years, it is important to use a pure steroid tracer especially if the tracer is to be added as an internal standard to determine recovery. Radioactive steroids should be purified at regular intervals; this should be monthly for tetra-labelled steroids, such as (2,4, 6, 7-^{3}H) 17β-oestradiol, and

every two months for other compounds (Brenner, Guerrero, Cekan & Dicz.falusy, 1973).

(1,2-^{3}H) Progesterone

Paper chromatography

1 ml of the stock solution of 25 µCi/ml in ethanol was purified by a Bush P10 paper chromatography system of petroleum ether (80° - 100°C)/ methanol/ water (100/70/30). The paper (Whatman No.1) was spotted with 50 µg of unlabelled progesterone and with the labelled steroid. The chromatogram was left to equilibrate overnight and was run for 3-4 hours at room temperature.

The strip containing unlabelled steroid was stained using the Zimmermanns reagent and the strip containing the radioactive band was passed through the Tracerlab 4π scanner, with butane;helium; 1.3;98.7 used at 5 lbf/in^2. The radioactive scan was aligned with the paper strip and the corresponding radioactive band removed, cut into small pieces and eluted with 10 ml ethanol. The ethanol was decanted and the paper was further washed with 5 ml ethanol.

The R_f value for progesterone was 0.84, and the estimated mean recovery was 71% (n = 5). A solution of ^{3}H-progesterone purified by paper chromatography 14 weeks previously was repurified using the same system. On the scan there were no apparent peaks due to impurity. This could mean that there was no deterioration in the label, or that the impurities were non-radioactive and therefore not detected.

Column chromatography using Sephadex LH-20

Since 1970 Sephadex LH-20 has come into use as a separation medium for steroids (Murphy, 1970). Sephadex LH-20 is a dextran gel with both hydrophilic and lipophilic properties, and separation depends upon gel filtration, adsorption and partition.

Although benzene/methanol (95/5) is the usual solvent system for progesterone (Youssefnajadian et al., 1972) it was decided on safety grounds to avoid benzene wherever possible. About 1g of Sephadex was soaked in approximately 10 ml of the solvent system (dichloromethane/methanol, 95/5) overnight. The excess solvent was then decanted and the remaining slurry poured into a Pasteur pipette which had been prerinsed with the solvent system and blocked at the narrow end with a glass bead (3mm diam). When the gel had reached a height of 7.5 cm. it was washed with solvent and a glass wool disc (the diameter of cork-borer size 3) inserted on top. The columns were kept on the cool-tray during use since separation deteriorates above 20°C (Murphy & d'Aux, 1975) ^{3}H-progesterone (10μCi) was added to the column in 0.2 ml of solvent mixture and allowed to drain into the column. Elution was set up by adding set volumes of solvent to the column, allowing this to drain through the column into a collecting tube, then transferring the whole column to the next collecting tube and repeating the process. The advantage of this method is that expensive columns with stopcocks or fraction collectors are not necessary.

10 μl of each fraction was counted on a scintillation counter. The fractions corresponding to the peak of ^{3}H-progesterone were pooled, evaporated, and redissolved in 10 ml of ethanol. The column was discarded after one use.

The elution pattern is shown in Fig. 2.1 . The recovery of ^{3}H-progesterone was 86%.

Conclusions

Both systems, paper and column chromatography, provided a purified label which was sufficiently pure to use in internal standard recovery experiments and in radioimmunoassay. However, paper chromatography has two disadvantages, in that paper residues

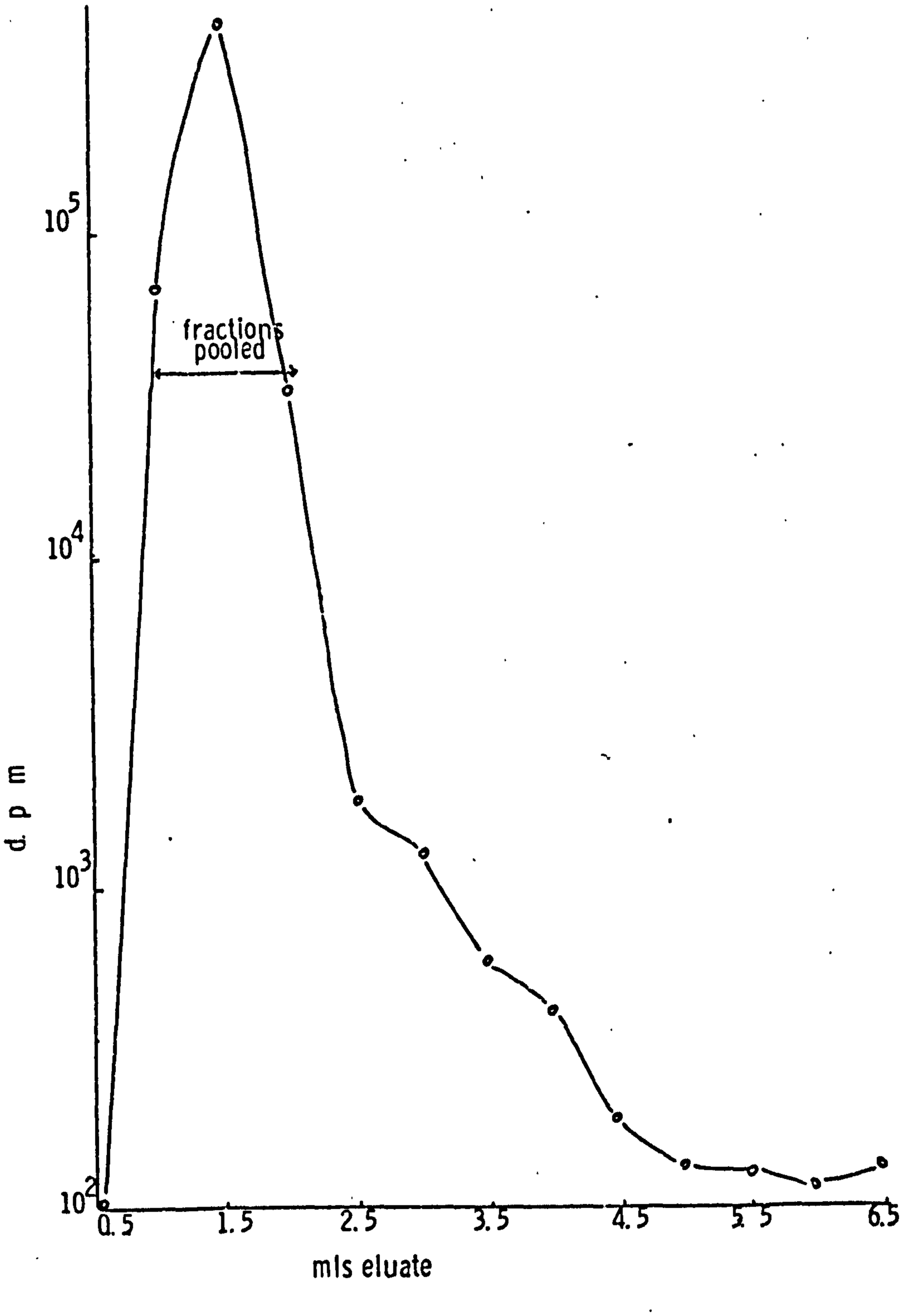


Fig. 2.1 The elution pattern of (1,2-^{3}H) progesterone from a column 7.5 cm in length, packed with Sephadex LH-20 in the solvent system dichloromethane/methanol, 95/5.

can be seen in the final eluate, indicating possible interfering substances, and in the length of the chromatography and detection procedures. Columns of LH-20 require about two hours and the product is free of apparent impurities since assays using this label were satisfactory. In the dichloromethane/methanol system the progesterone is eluted very quickly, which raises a few doubts as to the separating powers of the system.

(1,2-^{3}H) aldosterone

Although paper chromatography is the usual method for the purification of ^{3}H-labelled aldosterone, (Ito et al., 1972) a separation system on Sephadex LH-20 columns has been suggested by Murphy(1971) and such a method has now been used to purify the label (Waldhausl, Haydl & Frischauf, 1972). Both methods were compared.

Paper chromatography

The chromatography paper, Whatman no. 1, was washed overnight in a chromatography tank by eluting the paper with 200 ml redistilled methanol, and 10 µCi of ^{3}H-aldosterone (0.7 µg) was applied, in 0.1 ml ethanol to paper strips together with 50 µg unlabelled aldosterone. The solvents, benzene/water/methanol, 2/1/1, were equilibrated and the chromatograph run according to the method of Bush(1961), keeping the tank in darkness throughout the procedure. The radioactive band was located as described for ^{3}H-progesterone and the purified (1,2-^{3}H) aldosterone was eluted from the paper overnight with methanol, then with 2 ml of 90% methanol and finally with 3 ml methanol.

The R_f value for aldosterone (labelled and unlabelled) was 0.33 which compares with a value of 0.3 quoted by Gray, Greenaway & Holness, 1961) The recovery of radioactivity from the aldosterone band, as determined by scintillation counting, was 80%, 82% and 22% on three separate occasions, and since there was no evidence in the last run of high impurity, apparently the aldosterone was lost, underlining

one of the disadvantages of paper chromatography, in that the lengthy procedure tends to promote losses of labile steroids. Another disadvantage is the health hazard of using benzene, although toluene is a possible alternative.

Column chromatography

It was decided to try miniature columns in Pasteur pipettes for the chromatography step, and one gram of Sephadex LH-20 was soaked in 20 ml of dichloromethane/methanol, 98/2 (Murphy, 1971) and a column prepared to a height of 7.5 cm as described on page 73 . 10 µCi (0.7 µg) of (1,2-^{3}H) aldosterone was applied to the column and fractions collected as described on page 73 . The fraction showing the peak of radioactivity, i.e. the fractions from 2.0-2.5 ml were redissolved in ethanol, and pooled to a volume of 10 ml. The recovery of radioactivity was 82% from this peak and the only impurity eluted was at 8-10 ml.

When the steroid from the column was used subsequently in an assay it gave initial binding of 65%, sensitivity of 5 pg(see page 80) and a blank of zero. When labelled steroid from a paper chromatographic purification was used in the same way the initial binding was 60% and the sensitivity was again 5 pg but the blank was not tested. It seems therefore that both methods provide a satisfactory label for the assay.

^{3}H-labelled-oestradiol-17β

(2,4,6,7-^{3}H)-oestradiol-17β was stored in a solution of 25 µCi/ml in redistilled ethanol and repurified every four weeks on a Sephadex LH-20 LH-20 column, 7.5 x 0.5 cm, using dichloromethane/methanol, 95/5 (Murphy, 1971). The fractions containing the main radicactive peak were pooled, evaporated to dryness, and redissolved in ethanol to give a solution of about 1 µCi/ml, which was stored at 4°C.

1.3.2. Unlabelled steroids

Progesterone

A standard solution of 1.00 mg/ml progesterone in ethanol was prepared and the purity checked by thin layer chromatography on a silica gel plate 60F254 in the solvent system acetone/chloroform 5/95. The least amount of steroid detectable, when viewed at 254 nm, was 0.2 µg. The system gave good separation from other steroids such as 17-hydroxyprogesterone, and progesterone was apparently pure, but the same solution was retested one year later and two impurities were seen, which is fairly surprising as progesterone is rather unreactive, with the most likely reactions occurring as hydroxylations at the C-3 or C-20 positions (Klyne, 1965).

Another feature of progesterone is its poor solubility in aqueous media, even when protein is present (Clark & Gurpide, 1972). Thus it is important that the initial standard solutions are in ethanol or toluene, both of which we found to dissolve progesterone equally well.

Aldosterone

The purity of aldosterone, obtained from Sigma and from the MRC Reference collection was checked by thin layer chromatography as above, using the system ethanol/chloroform, 8/92. The chromatographs were repeated after aldosterone had been in solution for 6 months and 9 months. At 6 months two small impurities were seen on the chromatographs of the MRC standard, with R_f values of 0.08 and 0.16, compared to the value of 0.23 for aldosterone. After this the Sigma standard was used and it was still apparently pure at 9 months, but subsequently new stock solutions were prepared every 6 months and stored at -20°C in ethanol.

Oestradiol-17β

17β-oestradiol was dissolved in redistilled methanol to form a stock solution of 1.00 mg/ml and stored at -20°C for up to 6 months.

2. RADIOIMMUNOASSAY

2.1 General Considerations

The technique of radioimmunoassay (RIA) was first reported by Berson & Yalow (1959) for the measurement of insulin, and it has since been applied to the measurement of many proteins and also low molecular weight haptenic substances such as digoxin, cyclic AMP and the steroids. As already described (Chapter 1) immunoassay is now the method of choice for most steroids, especially now that specific antisera are available and chromatography steps are largely eliminated and since only immunoassays can provide the necessary specificity, sensitivity and convenience for rapid repeated sampling.

As a liquid scintillation spectrometer was already available in this Department tritium-labelled antigens were chosen for all the assays. RIA is however a deceptively simple technique and great care must be taken to assess performance if the results are to compare with other methods (Fraser, Guest, Holmes, Mason, Wilson & Young,1975).

As it has been necessary to produce radioimmunoassays for progesterone and aldosterone for this project, an outline of the principles and reliability criteria of radioimmunoassay are given, followed by a detailed account of the development of the assays for these two hormones. The radioimmunoassay methods used to measure other hormones in this project are given more briefly.

Principles of RIA

RIA is only one of many techniques involving the principle of saturation analysis, that is:

$$p + q = pq + p$$

where p is the substance to be measured, q is the binding reagent (such as an antibody) and pq is the bound portion. If some labelled

antigen, p*, is present in the incubation, there is competition for the available binding sites on the binding reagent, and the amount of labelled p* present as pq will be a function of the concentration of unlabelled p. The proportion of labelled p* in either bound or free fractions can be determined and plotted as a function of p (the standard curve). The three basic requirements for saturation analysis are therefore: (1) a labelled version of p which is stable and which can be easily measured, such as an isotope or enzyme-label (Engvall & Perlmann, 1971); (2) a specific binding reagent, usually an antibody, as in immunoassay, or a plasma protein, as in CPB; and (3) a quick and convenient method to separate the free and bound fractions.

One of the problems arising from immunoassay has been the lack of agreement regarding the kinetics of the reaction. The antibody-ligand interaction is complex and the standard curve obtained is nonlinear. The abscissa of the curve is in most cases the dose variable (unlabelled p) plotted on a linear or logarithmic scale. The ordinate may be the bound fraction, the free fraction, or a combination of both. The bound to free ratio (R) is a linear function of the concentration of bound antigen (B). This, the Scatchard plot, may be used to estimate the concentration of binding sites on the antibody (via the intercept) and the affinity constant via the slope (Feldman & Rodbard, 1971).

Other commonly used ordinates are the bound to total ratio (B/T), and the ratio of bound to bound at zero dose of p, B/B_o, although these two curves are nonlinear. For the assays in this department it was decided to use the B/B_o response variable against the logarithmic dose variable for most applications. This has the advantages of being simple to calculate; of being linear over most of the curve; and having a fixed range of 0 to 1.0 for B/B_o, which simplifies comparisons between assays.

Criteria for the Assessment of the Assay

The reliability of the assay is determined by the criteria of the standard curve, and by purifications procedures carried out prior to the RIA. The criteria of the standard curve are in turn largely determined by the quality of the antiserum. An antiserum is characterised by titre, (optimal dilution), by affinity (or avidity) which is expressed as the affinity constant, and by specificity, which is determined by cross reactivity studies. The chief reliability criteria of the assay are described below.

(i) Precision

Precision is the amount of variation in the estimation of unlabelled hormone, X, and is dependent on the precision of the standard curve at each point.

For straight lines the standard deviation of the response B/B_0 can be used (Feldman & Rodbard, 1971). With nonlinear curves the precision is not consistent over the whole range of the curve, and for this reason the precision of plasma duplicates will vary depending on the point of interpolation on the standard curve. Hence when the precision is quoted the range of hormone concentrations should also be mentioned.

The precision of the assay is also dependent on the error in the estimation of recovery of the hormone after extraction. Precision is usually expressed as the coefficient of variation of duplicate plasma measurements.

(ii) Sensitivity

The sensitivity of the assay is determined by three variables (Abraham, 1974).

a) The least detectable dose on the standard curve, or detection limit. This approximates to twice the standard deviation of the zero dose

response (Midgley et al., 1969). The least detectable dose will depend upon the error in the determination of Bo and also upon the slope at Bo, although as pointed out by Ekins (1975) the slope is to some extent an artefact of the variables plotted.

b) The blank values. These are indicated by a decrease (positive blank) or increase (negative blank) in binding in samples with zero dose of unknown, for instance water blanks, The blank value should ideally be less than the least detectable dose. Negative blanks are perhaps more of a problem as they are difficult to quantitate.

c) The recovery of steroid after purification.

Nonequilibrium assays: It has been shown (Rodbard, Ruder, Vaitukaitis & Jacobs, 1971) that if equilibrium is not reached the sensitivity of the assay may be improved by delayed addition of labelled antigen after a preincubation of unlabelled antigen and antibody. This may be especially useful in some steroid assays where sensitivity is of prime importance, and in practice a pre-incubation step is widely used (Cameron & Scarisbrick, 1973; Emment, Collins & Sommerville, 1972).

(iii) Accuracy

Accuracy may be defined as the extent to which the mean of an infinite number of measurements agrees with the exact amount of the substance which is present (Midgley et al., 1969). In the case of steroid assay this is measured by adding known amounts of steroids to water blanks or ideally to plasma containing no endogenous steroid.

Other methods of testing accuracy involve exchange of samples between assay laboratories and also the comparison of results by different methods.

(iv) Specificity

Specificity may be defined as the extent of freedom from interference by substances other than those intended to be measured

(Midgley et al., 1969). In the RIA of steroids a particular problem affecting specificity may be cross-reacting steroids, and this depends upon the characteristics of the antiserum. The specificity of the antiserum is tested by cross-reaction studies and if necessary, interfering steroids may be removed by selective solvent extraction or chromatography.

Nonspecific binding may also occur due to lipids or plasma proteins. If a solvent extraction is not necessary to remove cross-reacting steroids, then heating the plasma prior to assay may remove non-specific interfering proteins (Hennam, Collins & Sommerville, (1973).

2.2 Development of the assay for progesterone

2.2.1 The production of antisera

Although some antiserum was kindly provided by Dr. B.J.A. Furr it was decided to raise some antisera in our own laboratory. This was facilitated by a kind gift of conjugate to progesterone- 11α -hemisuccinyl BSA from Dr. M.W. Johnson at Chelsea Hospital for Women. Antisera raised with conjugates of progesterone coupled to BSA at position 11 show less cross-reactivity than those coupled at other sites (Kohen, Bauminger & Lindner, 1975) and are now widely used.

Characterisation of the conjugate: The method used was that of Erlanger, Borek, Beiser & Lieberman (1957).

Progesterone- 11α-hemisuccinate (P-HS) was dissolved in Tris buffer, pH 8.4 (Sigma) to a concentration of 3.5×10^{-7}M and progesterone-11α-hemisuccinyl BSA (P-BSA) and BSA were made up to concentrations of 6×10^{-7}M. The ultraviolet spectra of the compounds was investigated on a scanning spectrometer (Unicam SP800) at wavelengths from 400 nm to 200 nm for all the solutions.

The spectra were plotted (see Fig. 2.2), correction was made for the baseline absorbance at 325 nm, and this value was subtracted from the maxima at 246nm . The resulting maxima were 0.16 for P-BSA and 0.49 for P-HS. The steroid/BSA ratio was then calculated according to Erlanger et al (1957). The resulting ratio was 26 molecules of progesterone to 1 molecule of BSA. This conjugate with a molar ratio of 26 fulfills the criteria of Midgley & Niswender (1970) who suggested that a ratio of greater than 20 ensures a high titre. The theoretical maximum is 60, the number of free amino groups available on the BSA molecule for condensation reactions.

Purification of the conjugate

30 mg of the conjugate was dissolved in sufficient water and introduced into a wetted dialysis tube. The tube was sealed at one end and dialysed against distilled water overnight. The dialysis residue was then freeze-dried (in the Chemical Pathology Department, St. Thomas's Hospital Medical School) and the lyophilised conjugate was kept at $4^{o}C$ before use.

Immunisation

There is no generally agreed method for the successful production of steroid antisera nor for the animal species to be used, although the rabbit is the preferred animal in most cases (Nieschlag, Kley & Usadel, 1975). Rabbits are cheap, fairly easy to handle and a convenient size for the regular collection of serum. Many different sites of immunisation have been described (Nieschlag et al., 1975), but one of the most common routes of administration is subcutaneous injection at multiple sites with booster injections at regular intervals (Youssefnajadian, Florensa, Collins & Sommerville, 1972). Antibody response is enhanced by the use of oily adjuvant, which allows the sustained release of immunogen over

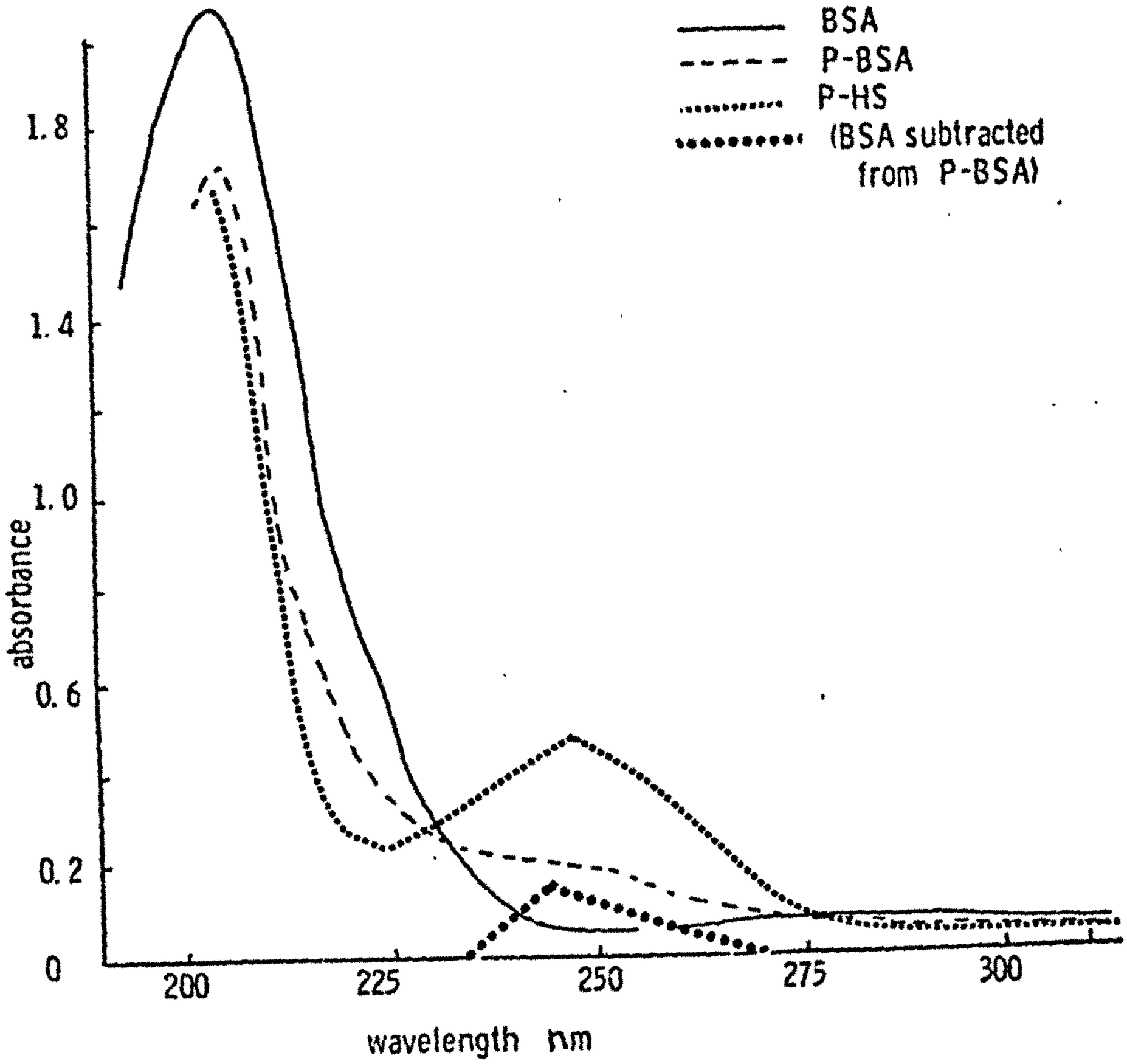


Fig. 2.2 Ultraviolet spectra of progesterone-11α-hemisuccinate (P-HS), 3.5×10^{-7} M, and progesterone-11α-hemisuccinyl-BSA (P-BSA), 6×10^{-7} M, and bovine serum albumin (BSA), 6×10^{-7} M.

several weeks, although the dose of immunogen may be less important (Hurn & Landon, 1971).

Four male New Zealand rabbits, one year old, were obtained several days before immunisation and kept in the animal house, St. Thomas's Hospital Medical School.

Eight mg of the conjugate was dissolved in 2 ml distilled water and 6 ml of complete Freunds adjuvant was added. The mixture was emulsified by vortexing for 10 min. Each rabbit was injected with a total of 2 ml solution (2 mg P-BSA) subcutaneously and intradermally on 10 sites over the back and haunches.

Booster injections of 1.5 to 2 mg conjugate in 1.5 to 2 ml of 50% incomplete Freunds adjuvant were given in the same manner at 8, 20, 28, 33 and 50 weeks.

Small test bleeds (1 - 2ml) were obtained from the marginal vein of the rabbits ear, which had been shaved, coated with a thin layer of vaseline, and nicked lightly with a needle. The blood was allowed to clot and then centrifuged at 1400 g for 10 min. The serum obtained was kept at $4^{o}C$ before testing the titre and cross-reactivity with 17α-hydroxy-progesterone (see below).

Larger volumes of blood were obtained by puncture of the ear vein in a similar manner except that a scalpel blade was used to incise the vein. Bleeds of 15 ml were collected at 18, 23, 32, 40 and 49 weeks. Half of the serum was kept at $4^{o}C$ with 0.1% sodium azide, and the rest was frozen at $-20^{o}C$ in small aliquots. Rabbit no. 1 died at 42 weeks but the other rabbits were anaesthetised and the blood harvested at 60 weeks, either by incision of the jugular vein (Rabbit 4) or by catheterisation of the carotid artery (rabbits 2 & 3), when 40 ml and 60-70 ml were obtained, respectively.

2.2.2. Assessment of the antiserum

The titre, cross-reactivity and affinity of the Furr antiserum (no. 465/5) and the St. Thomas' antisera were tested.

Titre: The titre is performed by incubating various dilutions of the antibody with a fixed mass of radioactively labelled steroid, in stated incubation conditions. The optimal dilution (titre) is likely to change if any assay parameters are changed, and for this reason the Furr antiserum (465/5) was re-tested, although details had been provided by Dr. Furr.

Working solutions of each antibody bleed were serially diluted in assay buffer, and incubated in the presence or absence of 500 pg unlabelled progesterone. The assay conditions are described in section 2.3.1.

The titre curve for the Furr antiserum 465/5 indicated that the 1/5000 working dilution gave 53% initial binding and a steep decline in counts if 500 pg progesterone was added, and this was taken as the optimal titre. As 0.25 ml of this solution is taken for each incubation, and diluted to 0.5 ml for the final assay volume, it is difficult to compare the titre with that of other workers who use different assay volumes.

The titre of the bleeds from the rabbits immunised with 11α-OH-progesterone-BSA was tested and the change in titre is shown in Fig. 2.3. The working titre of the bleed finally chose (B2S2) was 1/3000, equivalent to a final dilution of 1/6000.

Specificity: As an index of specificity the cross-reactivity of the test bleeds of the St. Thomas' antisera were tested using 17α-OH progesterone. The results are shown below in Table 2.1.

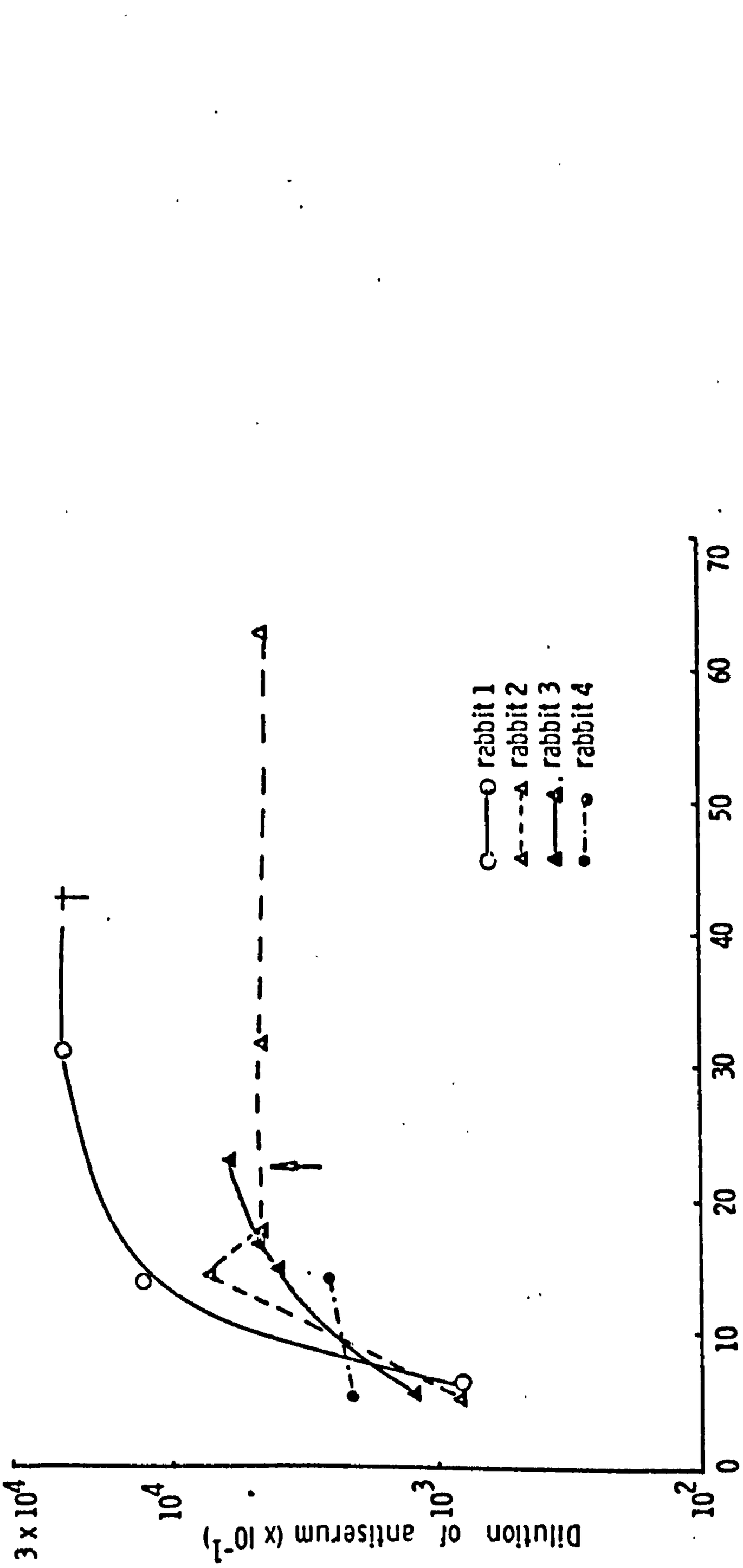


Fig 2.3 Change in titre with time in rabbits immunised with progesterone-11α-hemisuccinyl-BSA. The point at which Rabbit 1 died (†) and at which bleed B2S2 was taken (↑) are indicated.

Table 2.1

Cross-reactivity with 17α-OH progesterone

Rabbit	Cross-reactivity %	
	6 weeks	15 weeks
1	7.9	8.5
2	1.1	1.0
3	6.3	1.8
4	1.5	5.4
Furrs (from goat)	-	0.8 (8 weeks)

Rabbit 2 gave the highest specificity and a good titre at 15 weeks, and a further bleed from this animal at 23 weeks (B2S2) was tested with eight other steroids. The antiserum of Furr was also checked for cross-reactivity. The incubation conditions are described in 2.3.1. The results for B2S2 are shown in Fig. 2.4. The cross-reaction was calculated as:-

$$\frac{\text{pg progesterone causing 50\% drop in binding} \times 100\ \%}{\text{pg cross-reactant causing 50\% drop in binding}}$$

The main cross-reactivities found were for 11α-hydroxy-progesterone, 33%, pregnanedione 3.6%, 17α-hydroxyprogesterone 0.3%, pregnanolone, 0.3%, and 20α-hydroxyprogesterone 0.2%. Other steroids had cross-reactivities in the order of 0.005% (cortisol and cholesterol) and 0.1% (oestradiol and testosterone). The specificity of the antiserum is similar to that of Furr (1973) and Spieler et al. (1972), who also used a conjugate to progesterone-11α-hemisuccinyl-BSA.

Cross-reactivities for synthetic progestogens were also low (1.6% for dydrogesterone and 0.5% for D.H.D).

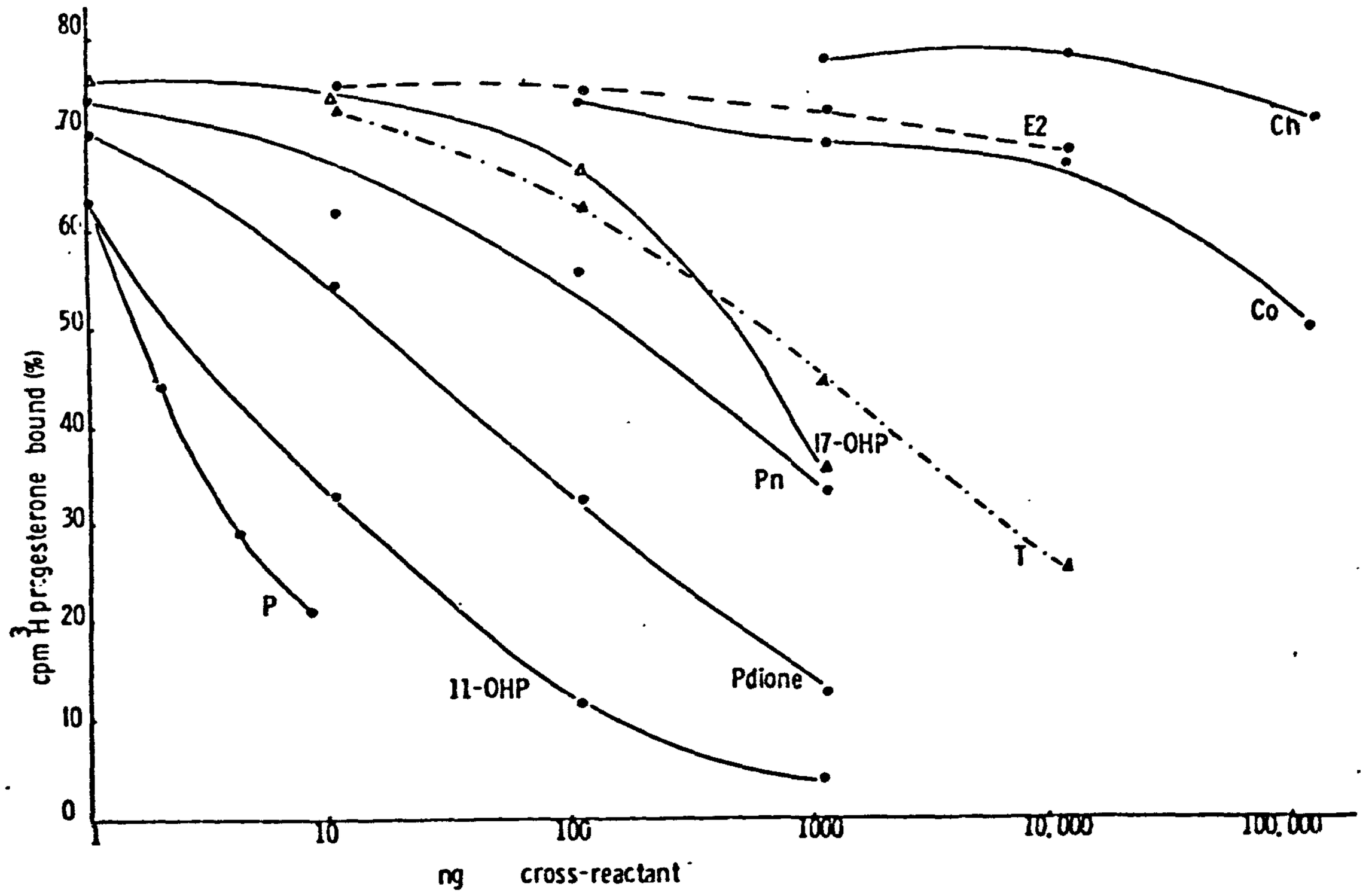


Fig. 2.4 Displacement of ^{3}H-progesterone from antiserum B2S2 by progesterone (P) compared to 11α-hydroxyprogesterone (11 - OHP), pregnanedione (Pdione), pregnenolone (Pn), 17α-hydroxyprogesterone (17-OHP), Testosterone (T), cortisol (Co), Oestradiol-17β (E_2) and cholesterol (Ch).

Affinity Constant: The affinity constants of the Furr antiserum and B2S2 were calculated using a Scatchard plot (see General Considerations). The affinity constant for B2S2 was derived from the linear portion of the curve (Fig 2.5), from the vertical intercept until an R value of about 1.0, since the effective affinity constant (K) governing the system is thought to be the K at B_0, that is the K at the vertical intercept (Ekins, 1971).

The K values obtained by this method were 3.7×10^9 l/mol for B2S2 and 3.0×10^9 l/mol for the Furr antiserum, which is in the mid-range of K values found by Midgley & Niswender (1970) for a variety of steroid antisera.

2.2.3 Purification of Plasma Prior to Assay

The objectives of a solvent extraction are to free progesterone from non-steroidal substances which may interfere from the assay, and to remove any natural steroids which cross-react with the antiserum (such as 17α-hydroxyprogesterone) or which occur in high levels in plasma (such as the corticosteroids). These conditions are met by the batch of petroleum ether with boiling point 30° - 60°C and hexane, which is preferred by some workers (Furuyama & Nugent, 1971).

Petroleum ether, bp 40° - 60°C, and hexane were redistilled, and 15 ml glass stoppered centrifuge tubes and 10 ml conical tubes were prerinsed with 1 ml of the solvent to be used. (1,2-^{3}H) progesterone, 5000 dpm/50 µl in ethanol was used as internal standard.

Plasma from non-pregnant women was thawed at room temperature, and fibrinous matter was removed by centrifuging the samples for 5 min at 1000g. It is thought that removal of this material increases final precision (D. Fahmy, personal communication).

0.1 to 0.4 ml of plasma were added to the tubes and diluted with water to a volume of 0.5 ml. 5000 dpm of ^{3}H-progesterone (in 0.05 ml

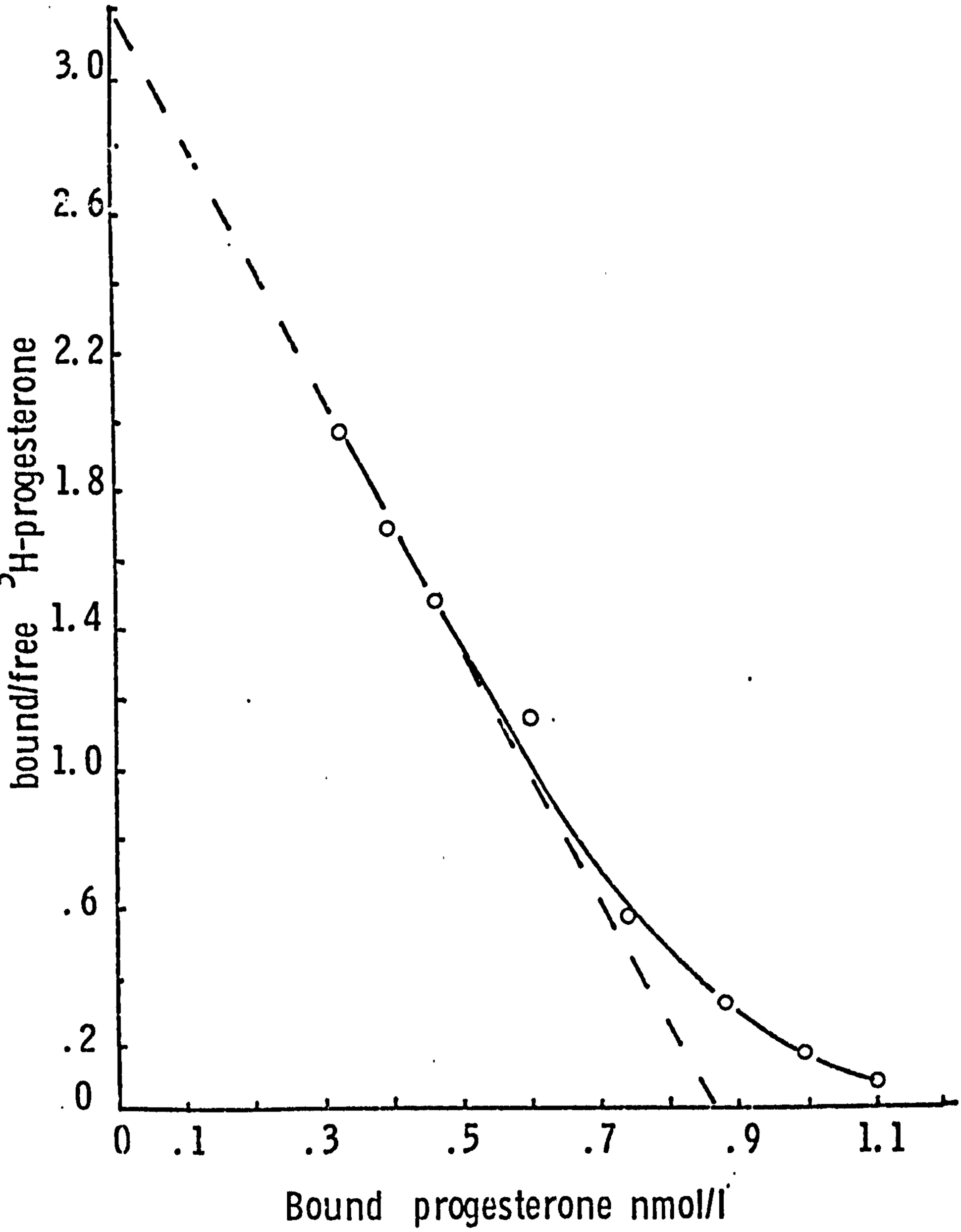


Fig. 2.5 Scatchard plot for serum B2S2. The effective slope (— — —) at the y intercept is 3.7×10^9 l/mol, which is the affinity constant, K.

ethanol) was added to all the tubes and to scintillation vials in triplicate to estimate recovery.

This step preceded the extraction by 30 min. to allow the added steroid in the tracer to equilibrate with the steroids already present. It is thought that this step is necessary as most steroids are bound to plasma proteins and will be extracted less readily than the free tracer, leading to an overestimate of the endogenous steroid extracted. Indeed, an equilibrium time of at least four hours has been recommended (Abraham, 1975).

After this incubation step 5 ml of solvent was added to the tubes and the tubes were vortexed for 30 seconds. They were then dipped into a mixture of carbon dioxide ice chips and ethanol for a few seconds. This froze the lower, aqueous layer, and enabled the solvent layer to be poured off into 5 ml tubes. The solvent was evaporated to dryness at 50°C in a water bath in a stream of compressed air. The extract was then redissolved in 0.4 ml ethanol and vortexed briefly, before aliquots were taken for scintillation counting.

No emulsions were formed using either of the solvents. The recovery of progesterone was 78 $\pm$ 2% (n = 4) using hexane and 85 $\pm$ 2% (n = 4) using petroleum ether. This was a significant difference ($P < 0.01$) when tested by Students 't' test. However there was no difference in the variability of extraction for the two solvents (Variance ratio test).

There was very little difference between the two solvents, either chemically or in their ability to extract progesterone. Therefore petroleum ether, which is much cheaper, was the preferred solvent in future assays. The equilibrium time for tracer and plasma was changed to 16 hours at 4°C. The average recovery of steroid in the subsequent assays is discussed in the section describing assay criteria.

2.2.4 Incubation with antiserum

Preparation and storage of reagents

Buffer: The RIA buffer was 0.1 M phosphate, pH 7.0 containing 0.1% gelatin according to the method of Furr (1973), although it was not found necessary to add sodium azide as the buffer was kept at 4°C and used within six weeks.

Labelled progesterone: (1,2-^{3}H) progesterone (58 Ci/mmol) and after December 1976, (1,2,6,7-^{3}H) progesterone (87 Ci/mmol) were purified as described above. A working solution of label was prepared about weekly by evaporating label in ethanol at 50°C in compressed air and adding buffer to give a solution of 20,000 dpm of ^{3}H-progesterone (32-48 pg) per 0.25 ml buffer.

Standards and Unknowns: The stock solution of 1 mg/ml was stored at -20°C for up to a year (see Section 1.3.2). Dilutions were made in ethanol at about six weekly intervals and kept at 4°C in glass stoppered tubes. The standards for assay were 0, 25, 50, 100, 250, 500, 1000 and 2000 pg in 0.1 ml volumes from the appropriate ethanolic solution, and these standards were added in triplicate to glass tubes, 10 x 75 mm. The standards were evaporated to dryness at 40°C in a vacuum oven attached to a vacuum pump prior to incubation.

The unknown steroid samples were treated in an identical way in that 0.4 ml ethanol was added to the dried extracts after petroleum ether extraction, as described, and 0.1 ml volumes were transferred to 10 x 75 mm tubes in duplicate and evaporated to dryness as above . Incubation then proceeded in the tubes.

Antiserum: Stock solutions of antisera were kept at 2-4°C in 0.1% sodium azide and 1/100 dilutions in buffer were prepared for regular use. For the preliminary experiments antiserum no. 465/5 was used at a working dilution of 1/5000 in buffer. For assays after 1975

antiserum No. B2S2 from this department was used at a working dilution of 1/3000 in buffer. The antisera were diluted immediately before use and volumes of 0.25 ml were used in the incubation.

Incubation Procedure

Exact conditions of incubation time, temperature and assay volume vary widely in the literature, although the pH is relatively noncritical (Rosa, Malvano & Rolleri, 1974). The incubation procedure used in this department was based on that of Furr (1973) but it was hoped to be able to shorten the overnight incubation and possibly to improve the sensitivity.

Standards, buffer, labelled progesterone, and antiserum 465/5 are described above. A series of six standard curves containing 0,500 and 1000 pg were set up. The antiserum and labelled progesterone solutions were mixed (1/1) before use. 0.5 ml of this solution was added to the standard tubes and mixed briefly on the vortex mixer, and the tubes were incubated for a range of times and temperatures. After each incubation 0.2 ml of dextran-coated charcoal (see 2.2.5) was added, and vortexed for 4 seconds. After 20 min at 4°C the tubes were centrifuged and 0.5 ml was taken for scintillation counting.

Fig. 2.6 shows the standard curves for incubations at room temperature (20°C) for 60 min., and at 4°C for 120 min. up to 16 hours. There appears to be a 'family' of curves with incubations over 2 hours, with no statistical difference (Students 't' test) between the points, except the zero dose. However, comparison of the overnight (4°C) and 1 hour (20°C) incubations reveals statistical differences at all points. The B_0 value increases with time, although a steady state does appear to be reached after 2 hours, which is presumably equilibrium. This is in agreement with the work of Rosa, et al. (1974) who found that equilibrum was reached within one to four hours for progesterone, oestradiol and aldosterone. Unfortunately the experiment in Figure 2.6. gave no information about the effects of incubation time on the detection limit.

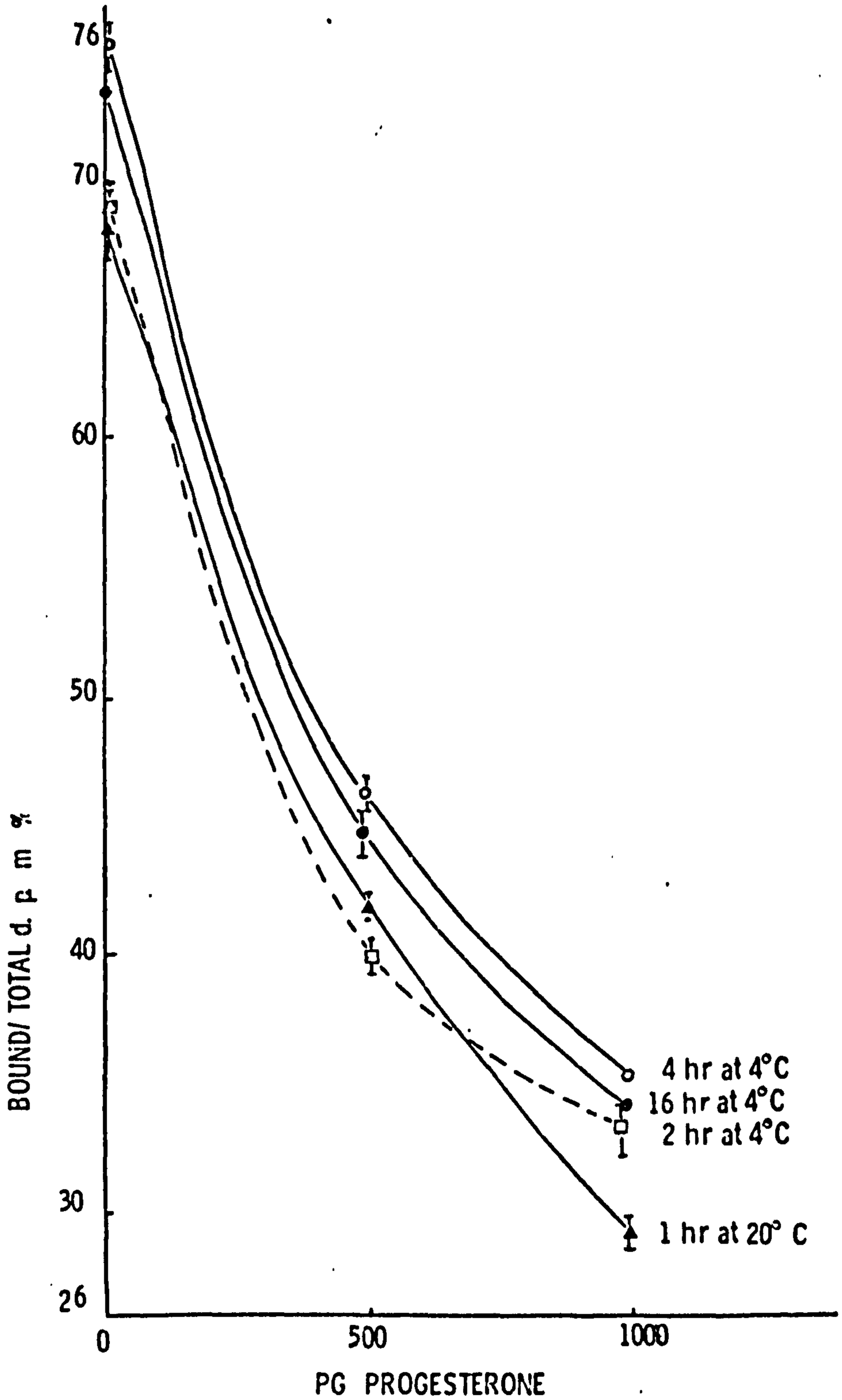


Fig. 2.6 Standard curve (± S.D.)(n = 3) for progesterone at different temperatures and incubation times.

One to two hours is the most convenient incubation time, although it may be necessary to reduce this on occasions. Although a pre-incubation step was not tested in the present experiment it was decided to add the antiserum separately, marginally before the tracer, for incubations of less than two hours, in the hope of improving the sensitivity.

There seems little effect of temperature in this assay, so that the only possible advantage of increased temperature would be to increase solubility of the dried extracts, and as this did not appear to happen in this assay it was decided to carry out further assays at $4^{o}C$ for one or preferably two hours.

2.2.5 Separation of antibody-bound and free steroid

Dextran is commonly used with charcoal for the separation of free and bound steroid as it is thought that dextran might act as a molecular sieve, excluding the bound complex from the charcoal (Herbert, Lau, Gottlieb & Bleicher, 1965). However Binoux & Odell (1973) have shown that dextran is unnecessary in some assays, but they recommend that conditions should be optimised for each assay method.

The effect of dextran in the separation of free and bound progesterone

A solution of 5% charcoal (C) in buffer was made up and dilutions were prepared, shaking the solutions thoroughly before pipetting, and using a wide bore pipette. The dextran-coated charcoal (DCC) was made up in a similar manner, starting with a stock solution of 5% charcoal, 0.05% dextran buffer. Before use all solutions were shaken thoroughly and they were stirred continually at $4^{o}C$ while in use.

The assay solution was prepared by mixing equal volumes of 465/5 antiserum dilution and ^{3}H-progesterone solution to give a volume

of 0.5 ml, in 10 x 75 mm assay tubes ("bound" progesterone, PAS) and also solutions of ^{3}H-progesterone only were added to a series of tubes and diluted to 0.5 ml with buffer ("free" progesterone, or antibody blank). 0.2 ml volumes of C and DCC were pipetted, using a Pipetman P1000, into tubes containing both free and bound steroid. The tubes were vortexed briefly and kept at 4°C for 20 min. and then centrifuged at 4°C for 10 min. at 2000 rev/min (1200 g) in a Mistral 4L.

Supernatant volumes of 0.5 ml were removed using a Selectapette not more than 10 minutes after centrifugation, and placed in scintillation vials. The vials were counted on the Packard counter. The total counts in the supernatant of both 'free' and 'PAS' solutions was counted. The percentage of counts in the supernatant could then be calculated, and the percentage of counts adsorbed by the charcoal obtained by subtraction.

Results: (a) Adsorption of free steroid

Dextran-coated charcoal adsorbs more than 95% of the free steroid at concentrations of 0.1% or more (Fig. 2.7). Charcoal alone is not efficient until a concentration of 0.25% is reached.

(b) Separation of bound and free steroid

Fig. 2.7 shows the percentage of free ^{3}H adsorbed by the charcoal where bound steroid is also present. DCC adsorbs 40% of the label, indicating that 60% is bound to the antiserum. However at concentrations above 1% the level of ^{3}H adsorbed by DCC rises, indicating that the bound steroid is being 'stripped' from the antibody complex. If C alone is used at concentrations where it adsorbs more than 90% free steroid, i.e. above 0.6% the level of apparent free steroid adsorbed increases more sharply.

It is not surprising that these findings differ from those

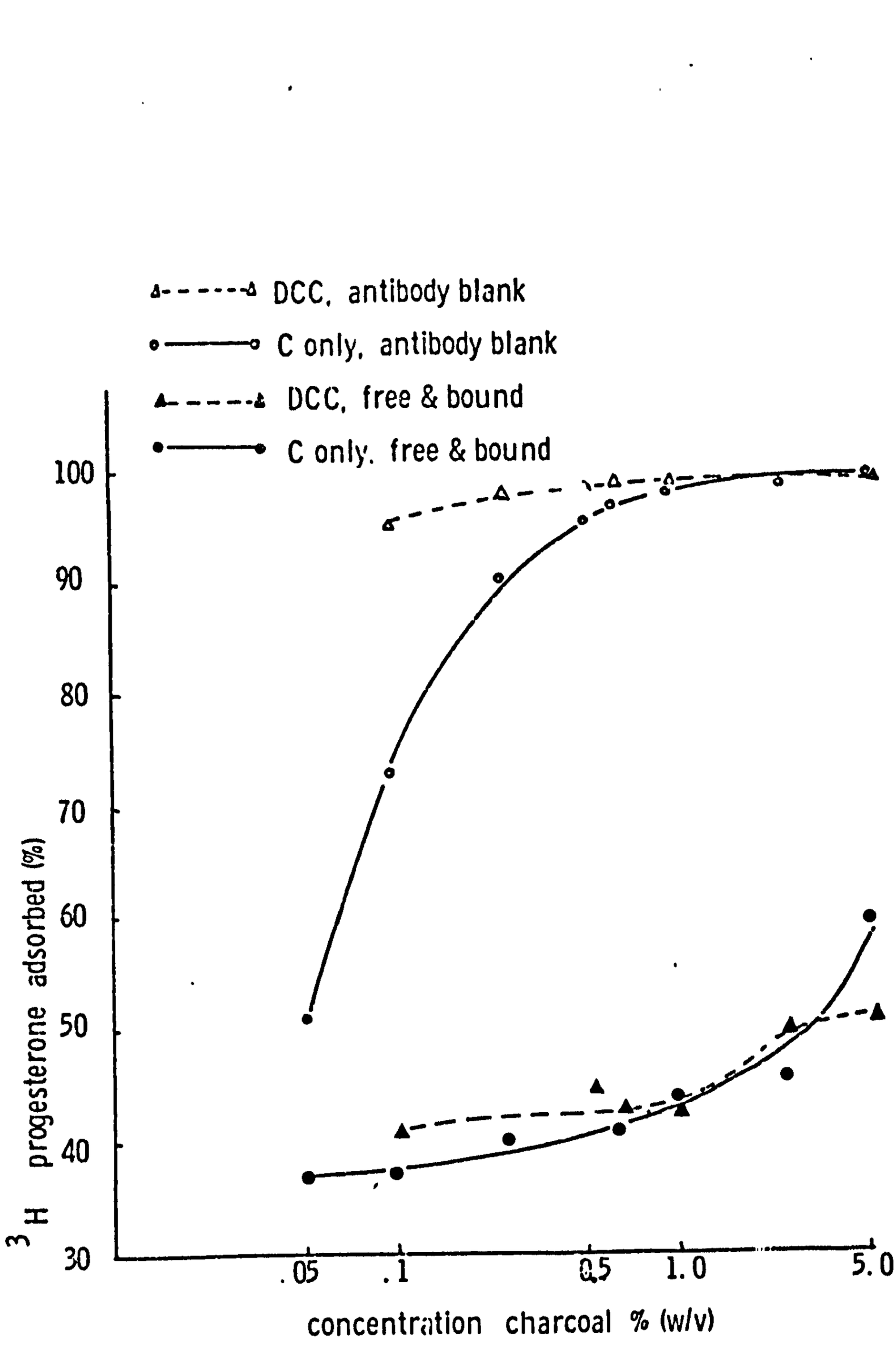


Fig. 2.7 The effect of increasing concentrations of charcoal (C) and dextran-coated charcoal (DCC) upon the percentage of ^{3}H-progesterone adsorbed, in the presence (▲,●) and absence (△,○) of antibody.

of Binoux & Odell (1973) since they were studying a protein-hormone assay with much higher protein content than is provided by additives in the buffer, such as BSA or gelatin. Indeed, it seems that the presence of dextran in the separation is advantageous for several reasons. Firstly, although both systems adsorb 97% or more of the free steroid (i.e. an antibody blank of 3%), DCC is efficient at lower concentrations, and over a greater range. This may be important as charcoal solutions are very heterogenous and fluctuations in concentration will occur during pipetting. Secondly, it appears that charcoal alone 'strips' the bound steroid more readily. This might give rise to several anomalies in the assay, such as positive blank.

The effect of incubation time with DCC

The incubation and separation procedures are described above. The only change was an increase in the concentration of label to 10^5 dpm/tube, because very low counts were expected in the supernatant of some tubes. The concentration of DCC was 0.625% charcoal, 0.00625% dextran (w/v) in buffer. All the incubations took place at $4^{o}C$.

Fig. 2.8 shows that in the antibody blank tubes the concentration of ^{3}H adsorbed rose from 98% at 5 min to 99.2% at 25 min, with a plateau after this. When bound counts were present a similar pattern was seen but counts in the DCC started to rise after 45 minutes. All the free steroid is adsorbed by this time, as indicated by the antibody blank, so that any increase in adsorption represents stripping of the bound complex. Therefore the incubation time for DCC with progesterone should be between 25 and 45 minutes.

2.2.6 Experiments to remove blank effects

From time to time both negative and positive blanks were present in the assays, and these were investigated.

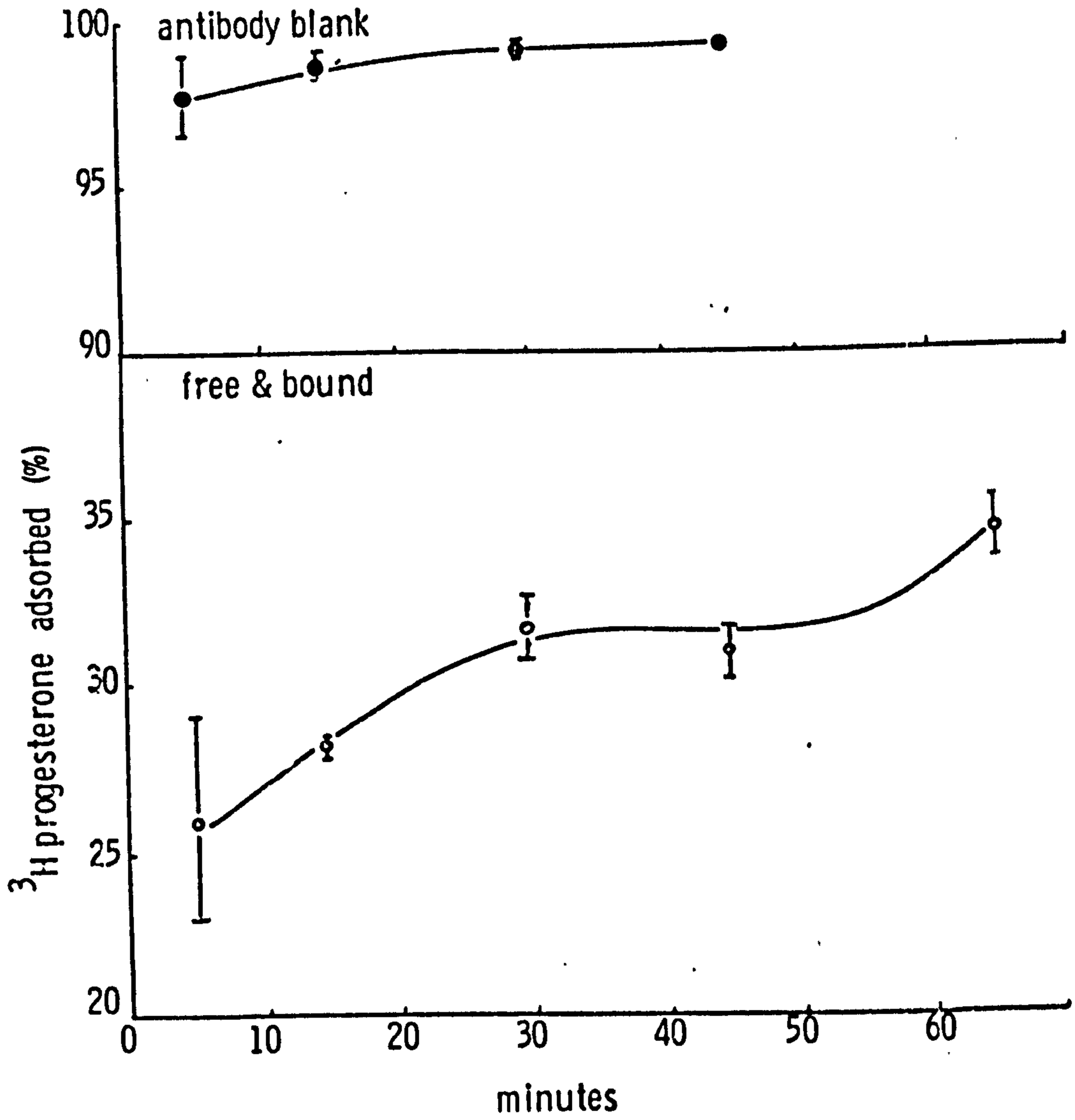


Fig. 2.8 The percentage (± S.D.) of ^{3}H-progesterone adsorbed by dextran-coated charcoal as a function of time, in the presence (o) or absence (•) of antibody. (n=3 at all points).

Effect of petroleum ether on the standard curve

Two standard curves were set up (see above). In the second set 5 ml of petroleum ether (40-60°C) was added to the tubes and evaporated to dryness in the vacuum oven before addition of the standards. The two curves are shown in Fig. 2.9. It appears that petroleum ether suppresses binding at up to 100 pg standard but it has the reverse effect at higher concentrations. Indeed, when this experiment was repeated petroleum ether caused higher binding at the doses of steroid below 100 pg and no effect at higher temperatures.

This demonstrates the unpredictable and variable effect of petroleum ether residues on the standard curve, which may give rise to either positive or negative blanks. As the effect may not be consistent over the whole standard curve, the blank (if positive) should not be subtracted from all the readings, although some authors have advocated this (Abraham, 1974). If the blank effect was less than the detection limit of the curve it was ignored. If blanks were a persistent problem for a while (probably due to a particular solvent batch), then some petroleum ether was added to the standards to compensate.

Investigation of negative blank

It is possible that a negative blank is caused by an substance which interferes with the adsorption of free steroid by the charcoal to give a high 'antibody blank'. This can be tested by measuring the adsorption of ^{3}H-progesterone by charcoal after mixing with plasma residues.

Assay conditions have been described previously. Volumes of non-pregnancy plasma (0.4 & 0.2 ml) were diluted to 0.5 ml with water, and extracted as described above. Extract residues were re-

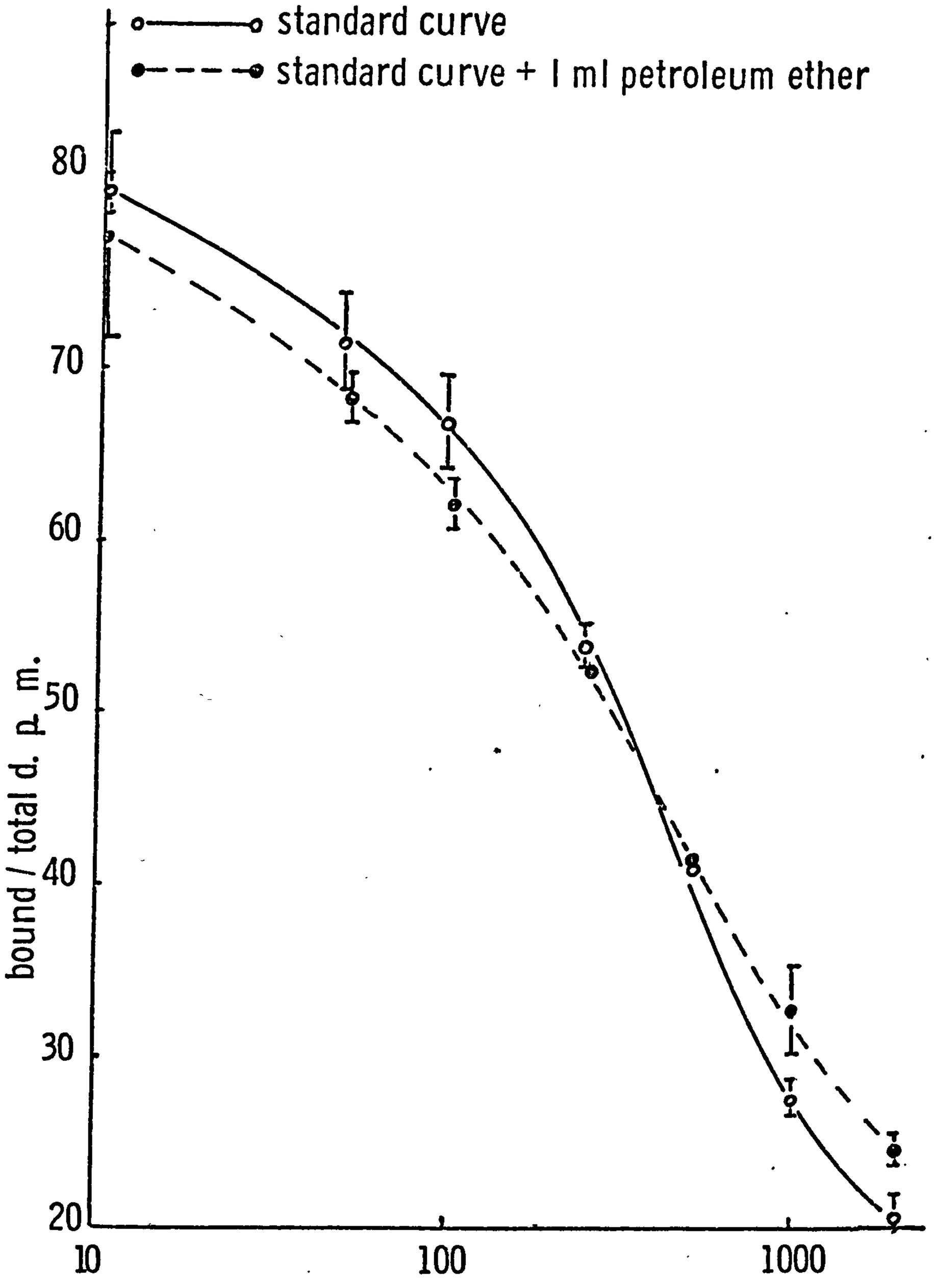


Fig. 2.9 The effect of the addition of 1 ml of petroleum ether, 40°-60°C, on the standard curve (± S.D) for progesterone (n=3 for all points).

dissolved in ethanol and 0.1 ml volumes transferred to assay tubes and evaporated to dryness. 0.5 ml of a buffer solution containing 20,000 dpm of ^{3}H-progesterone was added to the tubes and DCC was added as above.

The level of antibody blank was 0.8% (n = 3) in the tubes containing 2 ng standard, and it was 0.75% (n = 3) in the tubes containing the plasma extracts. It seems that interference with the separation system is not a cause of the negative blank. Many other mechanisms have been suggested which may account for the apparently higher binding in these blanks, such as interference with the tertiary structure of the binding protein (Pizarro & Kolanowski, 1972). This seems quite likely as the steroid-protein binding may be hydrophobic in nature (Rosa et al., 1974) and would therefore be susceptible to non-polar substances, such as lipids, left in the dried residues of organic solvents. However blanks were rarely a problem (see page 105) and when they did occur they were raraly outside the confidence limits of the B_0 values

2.3 The Final Progesterone Assay

2.3.1. Summary of procedure

The final working assay is summarised in the form of a flow sheet in Fig. 2.10. The detailed procedures have already been described:-

0.2-0.5 ml plasma thawed at room temperature

Two different dilutions added to tubes.

Add 0.05 ml ^{3}H-progesterone (5000 dpm) in ethanol. Leave overnight at 4°C.

Extract with 5 ml petroleum ether, 40-60°C

Vortex, freeze lower layer. Decant solvent

Evaporate to dryness at 40°C in air. (manifold)

Add 0.35 ml ethanol and vortex

2 x 0.1 ml in assay tubes

0.1 ml into insert vials to estimate recovery. Count for 10 min. or 10,000 counts

3 x 0.1 ml of standard; 0, 25, 50, 100, 250, 500, 1000, 2000 pg in assay tubes

Evaporate to dryness at 40°C in vacuum oven

Add 0.25 ml of 1/3000 antiserum (B2S2)

Add 0.25 ml ^{3}H-progesterone (20,000 dpm) (25-50 pg)

Incubate 1-2 hours at 4°C.

Add 0.2 ml of 0.62% DCC. Incubate at 4°C for 25 min.

Centrifuge at 1200 g for 10 min. at 4°C.

Decant 0.5 ml supernatant into insert vials

Add 3.5 ml scintillation fluid.

Count for 10 min. or 10,000 counts

Calculate (B/B_0) values for standard curve and interpolate unknowns. Correct for recovery.

Fig. 2.10 Flow sheet for final progesterone assay

2.3.2 Assay criteria

The Standard Curve

The initial binding was a mean of 54 ± 10 (SD) % (n = 34) in the presence of 35 pg of ^{3}H-progesterone, and the mean B_{50} value was 341 ± 90 pg. The non-specific binding (antibody blank) was constantly less than 2% of the total counts.

Blank: Out of a total of 105 assays, there were 10 water blanks values that were higher than the initial binding B_0 plus one standard deviation. These were classified as 'negative blanks'. In these assays the mean quality control was 6.9 ± 1.8 ng/ml, which is not significantly different from the mean quality control of the 'positive' blanks (7.1 ± 1.4 ng/ml). However negative water blanks were reflected in negative plasma blanks, indicating that the effect of the negative blank was not parallel throughout the whole assay, and this would be expected (see Fig. 2.9). There were also 54 water blank values within one standard deviation of the B_0 value and 41 water blanks reading between 25 and 50 pg on the standard curve, although there were no blanks higher than this.

Sensitivity: The detection limit of the progesterone standard curve was taken as twice the SD of the B_0 value on the curve, and this value was a mean of 10 pg in our curves. In theory this means that the lower limit of sensitivity, taking 0.4 ml of plasma and an aliquot for assay of 25%, is 100 pg/ml. In practice the blank values were sometimes higher than 10 pg and the detection limit was usually taken as 25 pg. The corresponding sensitivity in plasma is 250 pg/ml.

Most authors quote a detection limit of 20 to 25 pg (Furr, 1973; Furuyama & Nugent, 1971; Brenner et al., 1973), although Youssefnajadian et al (1972) could detect 10 pg on the standard curve.

Precision: The interassay precision was a coefficient of variation (CV) of 19% for a plasma pool of 7.0 ng/ml (n = 52) and a CV of 13% for a pregnancy pool (n = 31). This compares with CV values of 15% (Brenner et al., 1973) and 13.7% (Furuyama & Nugent, 1971) for female luteal plasma pools.

The intraassay precision was 15.0% (CV), (n = 5) for values less than 2 ng/ml, 9.5% (n = 5) for a value of 4.5 ng/ml, and 8.0%, (n = 5) for a value of 12.5 ng/ml. The corresponding precision by Furr (1973) is 5.1% for a value of 0.8 ng/ml and 6.8% for a value of 3.2 ng/ml.

For the entire method the mean CV was calculated for all the plasma values obtained. As each plasma sample was taken at two dilutions the pairs were not strictly duplicates, and it is difficult to make comparisons with other papers. Using a competitive binding protein assay and measuring duplicate pairs, Johansson (1969) found a CV of 14.5% for values of 5-10 ng/ml, which was calculated using the formula:

$$CV = \sqrt{\frac{\Sigma (d^2)}{2n}}$$

where d is the percentage difference between duplicate values and n is the number of duplicate pairs (based on Snedecor, 1956). According to this formula the mean coefficient of variation of our values was 18% in the range 0-2.0 ng/ml (n = 28), 10% in the range 2.0-10.0 ng/ml (n = 46) and 15% in the range 10.0-20.0 ng/ml (n = 38).

Accuracy: Known amounts of steroid were added to water and to follicular phase plasma. The result is shown in Table 2.2 The endogenous steroid in the plasma was subtracted from the result.

Table 2.2. The recovery of progesterone added to water and to follicular phase plasma, corrected for endogenous levels.

(x) Added steroid	WATER (y)			PLASMA (z)		
ng	steroid measured ng	SD	n	steroid measured ng	SD	n
0.25	-	-	-	0.262	.034	5
0.5	0.505	.12	7	0.481	.071	5
1.0	1.05	.22	52	1.059	.065	5
2.0	2.18	.42	8	1.920	0.168	2
4.0	3.76	-	1	-	-	-

These values gave a regression equation for the water samples of:

$y = 1.07x + 0.93$ $\quad r = 0.995$

and for the plasma samples: $z = 0.97x + 0.02$ $\quad r = 0.99$

Another measure of accuracy was to exchange samples with another laboratory. This was arranged with Dr. Margaret Swain at the Imperial Cancer Research Fund, who used a competitive protein binding assay. The following equation was obtained for pregnancy plasmas:-

(our result) = 1.10 . (Dr. Swains result) + 6.82 (n = 11)

r = 0.89

An exchange was also carried out with Dr. D. Fahmy at Cardiff who was using an RIA similar to our own. The equation obtained for a comparison of female luteal phase bloods was:-

our result = 1.03 (D. Fahmys result) + 1.78 (n = 13)

r = 0.92.

Recovery: The procedural losses were estimated by calculating the recovery of some internal standard added to the samples before extraction. The mean recovery in one typical assay was 71.7 $\pm$ 2.5% (n = 46). This compares with the mean recovery found by Furr (1973) of 74.5 $\pm$ 3.1%, although it may be possible to obtain recoveries consistently above 90% if the right batch of petroleum ether is found

(Cameron & Scarisbrick, 1973).

2.4 Development of the Assay for Aldosterone

2.4.1 Antiserum

The antiserum to aldosterone- 18, 21-dihemisuccinate BSA was originally prepared by Dr. R. Haning in the laboratories of Dr. & Mrs. J.F. Tait. The conjugate was prepared by the method of Erlanger et al. (1957), and the conjugation and immunisation procedures are described by Haning, McCracken, St. Cyr, Underwood, Williams & Abraham (1972). The antiserum, batch No. 088, was already diluted 1/100 when received, and 1 ml. aliquots were frozen except for the working solution, which was kept at $4^{o}C$ with 0.1% sodium azide (BDH) added. The titre was tested using the assay conditions and reagents described in later sections.

Titre: A titre of $1/(5 \times 10^{5})$ was found to bind 50% of 50 pg of labelled aldosterone, and to give a B_{50} value of 50 pg.

Specificity: Haning et al (1972) found that there was less than 1% cross-reactivity with cortisol, corticosterone, cortisone, testosterone, dehydroepiandrosterone, progesterone, and oestradiol -17β. It has been shown by Haning and co-workers (1972) that the cross-reactivity does not alter with different assay conditions, at least for this antiserum.

However some of the cross-reactivities were retested by us and the following values were found: cortisol .002%; corticosterone, 0.2%; dehydroepiandrosterone, .005%; DOC .06% tetrahydrocortisol .001% and 18 -DOC .01%.

It was found that if the chromatography step in the assay was excluded the apparent value in the sample was 214 ± 32% (n = 2) of the value found after LH-20 chromatography. The value found after a charcoal purification, as described on p.115, was 121 ±

23% (n = 3) of the value found after chromatography. Although this is not an absolute test of specificity it does show that a purification step is necessary and that the chromatography step is preferable to other methods.

Affinity Constant: The affinity constant was calculated from a Scatchard curve and found to be 2 x 10^{10} l/mol with a second slope at higher concentrations of bound antigen.

2.4.2. Purification of plasma prior to assay

While there is disagreement about chromatographic procedures for the purification of plasma, most authors employ an initial extraction step with dichloromethane(DCM).

Extraction step: Glass-stoppered tubes, of 30 ml and 15 ml capacity, were pre-rinsed with 5 ml dichloromethane before use. 0.05 ml of internal standard containing 9000 dpm of (1,2-^{3}H) aldosterone was added to the tubes and the volume made up to 2 ml with glass distilled water. Total counts were estimated by adding 0.05 ml of internal standard directly to the counting vials. Either 8 ml (to the 15 ml capacity tubes) or 20 ml (to the 30 ml capacity tubes) of dichloromethane was added and the tubes were vortexed for one min. The aqueous layer was removed using a Pasteur pipette attached to a small suction pump. The dichloromethane was evaporated to dryness in an air stream at 40°C in a water bath. When just a trace of solvent remained, the residue was dissolved in 0.2 ml of DCM/Methanol, 98/2 (this was to be the solvent system for the next stage in the purification). The recovery of labelled steroid was measured by counting volumes of the extracts by liquid scintillation spectrometry.

Recovery of internal standard after 30 minutes pre-incubation with the plasma was 67 ± 4% (n = 2) with 20 ml solvent and 55 ± 11%

(n = 4) with 8 ml solvent. If the pre-incubation step was omitted the recovery was 60 ± 1% (n = 2).

These recoveries are low in comparison to those of other workers; Poulsen et al (1974) found a recovery of 89± 5% using 10 ml DCM with 1-3 ml serum. The poor recovery in this experiment could be due to inadvertent removal of DCM while aspirating the upper layer, or it could be due to losses in the blowing down process as Poulsen et al (1974) used nitrogen and not air.

There was no statistical difference between recoveries with and without preincubation suggesting either that endogenous aldosterone is not bound to plasma proteins or that 30 minutes preincubation is not sufficient. There is some recent evidence for a specific aldosterone-binding protein in human plasma (Richardson, Nowaczynski & Genest, 1976) but the importance of this is not yet known.

It was decided to use 8-10 ml solvent (as the smaller tubes were more convenient) and to use nitrogen if at all possible in the blowing down of aldosterone solutions.

Further purifications of the plasma extracts

As most antisera are not specific enough to discriminate between aldosterone and the corticosteroids in a thousandfold excess, (see Chapter 1) it has been necessary until recently to perform a chromatographic step in the assay.

The first RIA reports used paper chromatography for purification of plasma extracts with antisera crossreacting in the order of 0.01% for cortisol. Bayard et al (1970) used the B5 system on Whatman No. 2 paper and Mayes et al. (1970) added an extra silica gel column after this. Ekins, Newman, Piyasena, Banks & Slater (1972) used the B5 system with SG81 paper.

Ito et al. (1972) first described a purification step using Sephadex LH - 20 on a 60 x 1 cm. glass column and using dichloromethane/methanol as the solvent. They reported that blank values were negligible. LH - 20 chromatography has been reported for other applications such as urine extracts (Waldhäusl et al., 1972) or to measure aldosterone and deoxycorticosterone in the same sample (Castro, Kutas, Jelen & Bartos, 1974). LH - 20 columns using water as the solvent have been described (Nowaczynski et al., 1974).

Other workers have tried to avoid the chromatography step. Farmer, Brown, Howard and Fabre (1973) oxidised aldosterone to the lactone and used an antibody specific to the lactone itself. Other workers have taken an immunological approach and have used an antibody twice, firstly to remove the aldosterone from the solutions in the bound form, then to re-extract and assay it (Gomez-Sanchez, Kem & Kaplan, 1973; Martin & Nugent, 1973). However these methods were tedious, and the only possible advantage over chromatography is the possibility of automating these techniques.

Highly specific antisera have been extensive purification unnecessary (see Chapter 1) but these antisera were not easily available to us. It was decided to try Sephadex LH - 20 first since it had already proved convenient, and also to test non-chromatographic methods of purification.

LH - 20 columns

Columns were prepared using dichloromethane/methanol, 98/2, as described in section 1.3.1. except that, to allow for a reservoir of 1 ml capacity, the columns were only 6.5 cm in height. Columns containing Sephadex LH - 20 which had been soaked in distilled water were also prepared. Volumes of (1,2-^{3}H) aldosterone containing 0.1 μCi (630 pg) were evaporated to dryness and redissolved in 0.2 ml of the solvent to be used on the column. The steroids were applied

to the columns and 0.5 ml fractions were collected until 15 ml. solvent had been eluted. (1,2-^{3}H) progesterone (0.6 µCi, 3.2 ng) and (1,2-^{3}H) cortisol (0.1 µCi, 0.4 ng) were also added to the columns to determine the elution pattern. They were mixed with ^{3}H aldosterone and run on the columns as before.

The flow rate for DCM/MeOH was 10 ml / h and for water it was about 5 ml/h. The elution pattern for both systems is shown in Fig. 2.11. These graphs are a composite of three different columns running aldosterone, aldosterone plus cortisol and aldosterone plus progesterone. No separate peak could be obtained for cortisol or progesterone on the water column, at least in the first 7 ml eluate. A sharp peak for aldosterone was obtained at 2.25 - 3.25 ml eluate on the DCM/MeOH column, with a peak for progesterone at 1 ml and for cortisol at 5 ml. The peak for aldosterone on the water columns was at 2.5 - 3.5 ml on the column, and at 3-4.5 ml on the second.

It is difficult to compare the elution patterns of other authors with our own as the column dimensions are all different. However an attempt was made to do this, assuming the principle of gel filtration, and calculating the volume of each column from the dimensions applied in the papers by applying the formula (volume = r^2 x height). The comparison is shown in Table 2.3.

TABLE 2.3. The elution pattern of steroids using the DCM/MeOH (98/2) system and different column sizes

Author	Volume cm^2	conversion factor (10^{-1})	PEAK ml Prog	Aldo	Cortisol
This study	1.3	1	1	2.7	5
Ichikawa et al (1974)	7.5	5.8	-	2.6	4.2
Daniel et al (1974)	15.1	11.6	1	2.0	3.1
Waldhäusl et al(1972)	19.7	15.1	-	2.3	3.7
Murphy (1970)	25.4	19.5	0.7	2.0	3.6

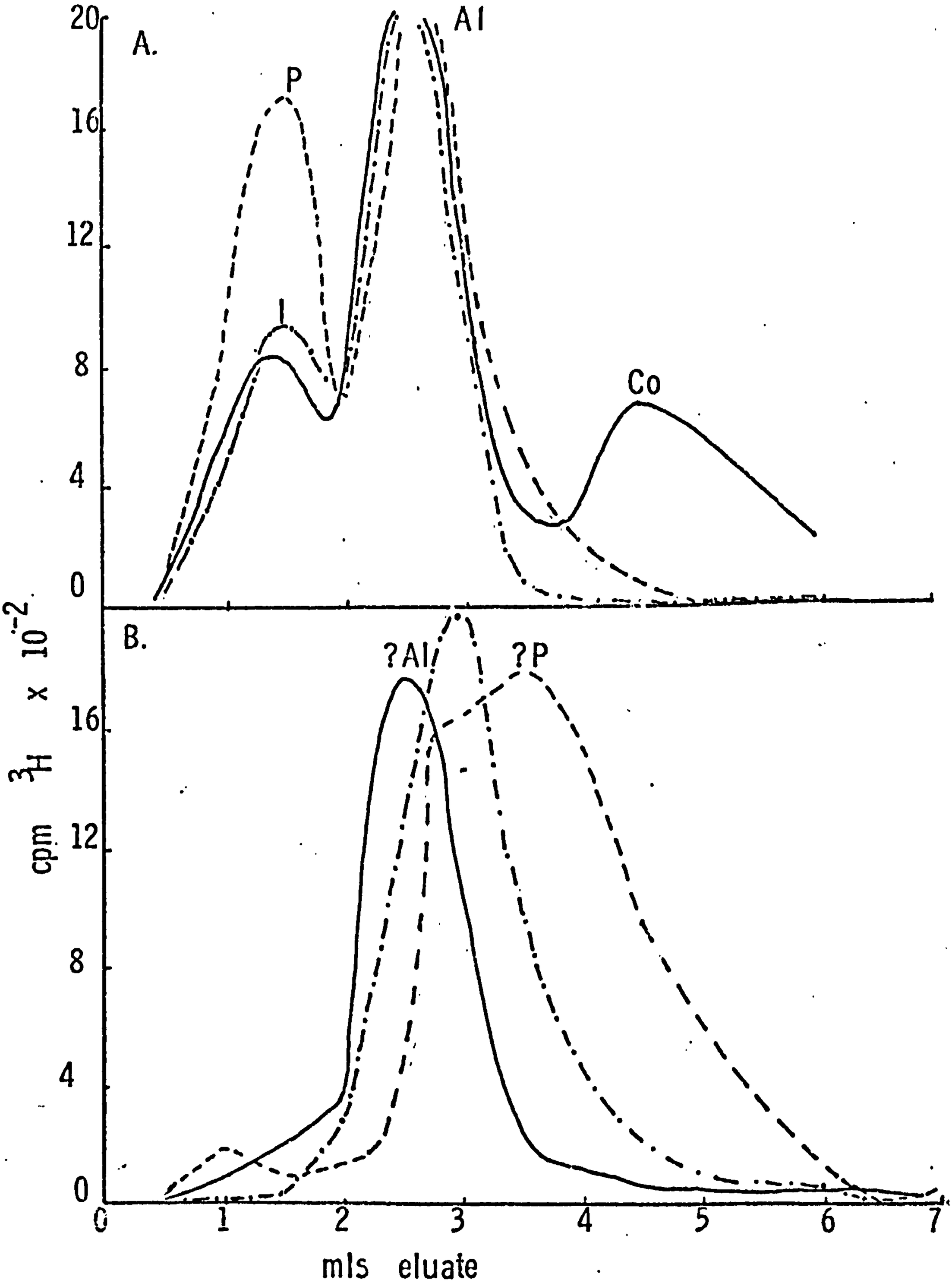


Fig. 2.11 Elution patterns of Aldosterone (Al) (—·—·), aldosterone plus progesterone (P) (– – –), and aldosterone plus cortisol (Co) (——) from columns 6.5 cm in length, packed with Sephadex LH-20 in two solvent systems; A, dichlorcmethane/methanol, (98/2) and B, distilled water. A small peak (I) was eluting in the same position as progesterone in solvent system A when aldosterone was run.

This is a very crude comparison, as other factors such as temperature and flow rate were not taken into account. But columns of different volume seem comparable in this way, and if anything, the smaller columns allow a relatively better separation. This encouraged us to proceed with the 'mini' columns further. As to the better solvent system, the dichloromethane/methanol system was preferable on two theoretical and two practical counts.

1. The separation between cortisol and aldosterone using the water system was not proved to be satisfactory despite the results of Nowaczynski et al (1974). There was no apparent reason for this.
2. The column profiles of the water columns were not reproducible. This is especially important if large batches of columns are to be run without checking the peak each time.
3. The water columns were slower to run, and nonpolar steroids such as progesterone were eluted rather late. This would mean that columns would require washing with very large volumes of water between runs.
4. An eluate in water would be difficult to evaporate to dryness in a short time.

Therefore, rather reluctantly in view of the unpleasantness of DCM, the DCM/MeOH columns were used in future assays and the water columns were discarded.

Calibration of the columns

The reproducibility of the elution pattern was tested, from column to column, and marker dyes which help to locate the steroid peak (Drewes & Kowalski, 1974) were investigated.

Columns were prepared as in Section 1.3.1. except that columns were 6.5 cm. in height. Three columns (A-C) were made 4 days before use and kept in a tightly sealed jar containing some solvent, at $4^{o}C$. Three weeks later another column (D) was made up freshly and run on the same day. The dyes were isatin and 1,4, diaminoanthroquinone which had been recommended by Ichikawa, Murata

& Shigiya (1974). Columns A-C were run simultaneously after 20,000 dpm ^{3}H-aldosterone in 0.2 ml eluate had been added to each column. The crystalline dyes were dissolved in a few drops of the solvent and added to the columns with the ^{3}H-aldosterone.

The elution pattern for the four columns can be seen in Fig. 2. 12. Although column D was prepared and run three weeks after A-C the fraction eluting aldosterone remains constant. The flattened peak and long 'tail' of column C could be due to Sephadex escaping from the column, since some of the gel was found in the collected fractions.

Isatin did not appear until after the main peak of aldosterone (Fig. 2. 12). 1,4diaminoanthroquinone itself separated into two dyes on the column. The stronger purple dye eluted at the same place as isatin, and weaker pink dye was eluted before the main peak of the aldosterone.

As the columns are reproducible from column to column and over time, it seems safe to use the columns without dye, so long as prior calibration of a few columns from the batch has been carried out. The recovery of ^{3}H-aldosterone from the columns is described in section 2.5.2.

Evaluation of charcoal precipitation technique to separate aldosterone from (bound) cortisol

Cortisol is largely bound to cortisol binding globulin, while aldosterone is largely free or bound loosely to albumin (Sandberg, Rosenthal, Schneider & Slaunwhite, 1966). Aldosterone can be partially purified by separating bound and free steroids in plasma, and Varsano-Aharon & Ulick (1974) have used charcoal to do this before assay with antiserum No. 088. We attempted this method in the hope that it might improve recovery and streamline the assay.

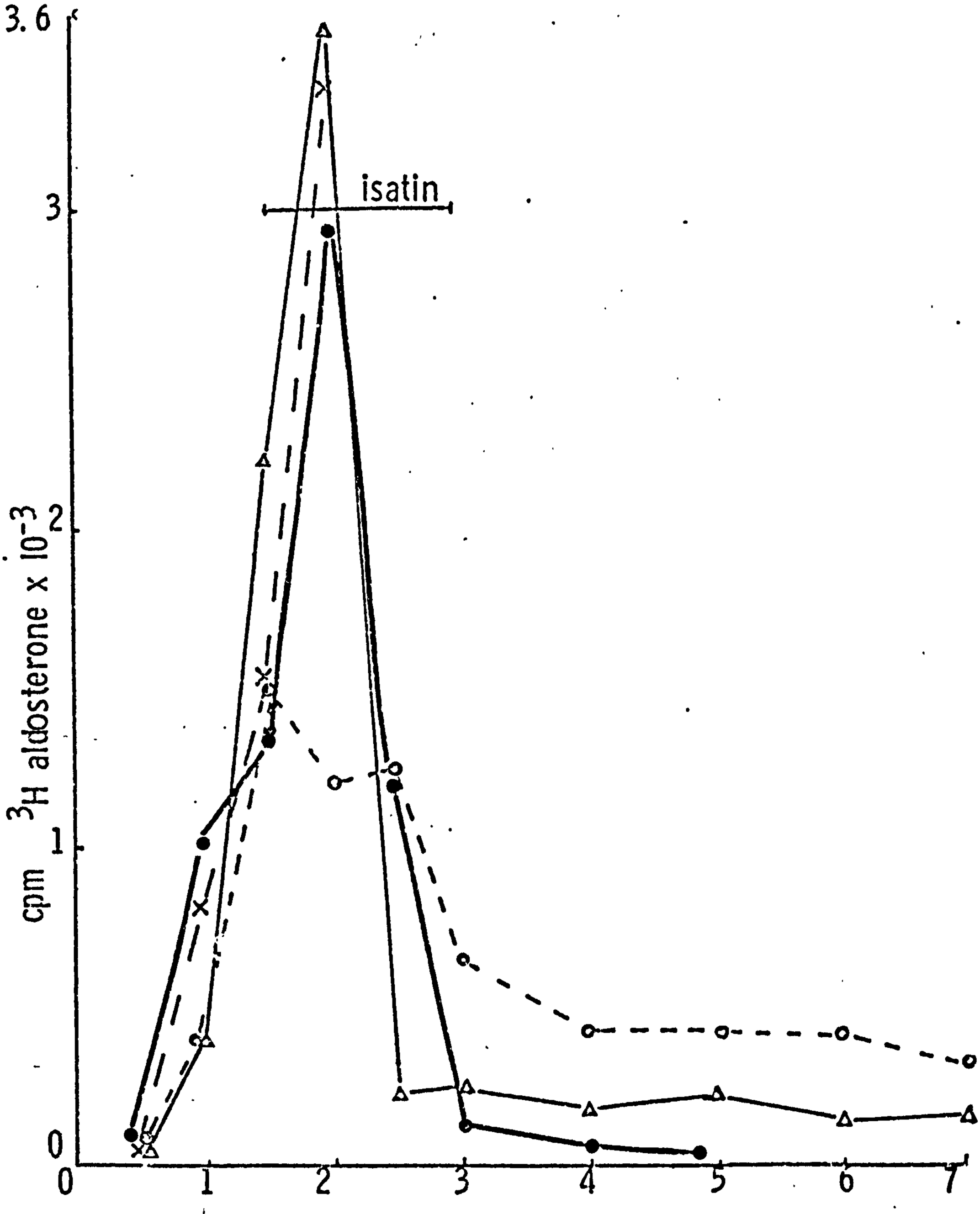


Fig. 2.12 Reproducibility of elution patterns from Sephadex LH-20 (DCM/methanol, 98/2); columns A (×—×), B (Δ—Δ), and C (○- - -○), packed and eluted together, and column D (●—●) packed and eluted three weeks later. The position at which the dye isatin is eluted is indicated.

The charcoal method of purification was adapted from the method of Varsano-Aharon & Ulick (1974). 3 gm of Norit A was 'washed' by vigorous shaking with 20 ml 0.1M phosphate buffer, and then centrifuged for 15 min at 1400 g. The buffer layer was removed and 50 ml buffer containing 60 mg dextran C was added. This was the DCC solution used to purify the plasma (solution A). Plasma samples from pregnancy, and water samples containing known amounts of steroid, were assayed. The plasma (0.2 ml) was diluted to 2 ml with water in 15 ml capacity stoppered centrifuge tubes, mixed with 6000 dpm ^{3}H-aldosterone, then left for 30 min at 4°C.

Charcoal purification: 0.5 ml of solution A was added to the samples and the tubes vortexed for 4 seconds. The tubes were centrifuged at 4°C at 1200 g for 10 min and the supernatant was removed and a 1 ml fraction taken for counting. The charcoal precipitate was washed with 5 ml buffer, the tubes vortexed and centrifuged again, and the buffer removed. The aldosterone in the charcoal was resuspended using 0.5 ml DCM, vortexed briefly, and a further 7 ml DCM added. It was found that only by this two stage process was the charcoal resuspended. The tubes were finally centrifuged as above and the DCM poured off. It was found that the charcoal did not precipitate as expected from the solvent, but that the DCM could be poured off cleanly. The DCM was evaporated to dryness at 40°C and the residue redissolved in 1 ml ethanol. Fractions were taken for assay and to estimate recovery by liquid scintillation counting.

LH - 20 columns: Identical samples were purified by dichloromethane extraction and column chromatography as described previously.

Assay: All the samples were assayed as described in 2.5.1.

The recovery of internal standard was a mean of 61 ± 6% (n = 18) for charcoal purification. The amount of aldosterone lost in the initial charcoal separation is low as less than 1% of the

radioactivity was found in the initial supernatant. Therefore the losses must occur because not all the free aldosterone is re-dissolved in the DCM.

The very low recovery of aldosterone in the bound fraction in the pregnancy samples is surprising since Varsano-Aharon & Ulick (1974) found that 15-30% of ^{3}H-aldosterone may be present in the supernatant and these figures may be higher in pregnancy when CBG is increased. The amount of aldosterone bound to CBG, although small, is variable and may be altered by changes in temperature and CBG concentration (Westphal, 1970).

The plasma values found after charcoal and column purification are shown in Table 2.4.

TABLE 2.4. Values found in the same plasma extracts after column or charcoal purification

Sample duplicates	COLUMNS		CHARCOAL		Mean Charcoal / Mean Columns %
	mean pg/ml	CV%	mean pg/ml	CV%	
Non pregnancy	243	26	343	29	141
Pregnancy (1)	474	10	595	20	126
Pregnancy (2)	588	4	567	19	96
50 pg	78	11	41		52
250 pg	234	26	62		26
blank	2.5		6.0		240

The blank values for both systems are satisfactory, but there is a worrying over-estimate in the plasma samples measured by charcoal purification in two cases, indicating some cross-reactivity still remaining. It is inferred that it is an overestimate by the charcoal method rather than an underestimate by the column method because the accuracy samples do not show the same trend. If there

were a systematic error in the column method causing an underestimate it would show up in all the samples presumably, whereas an overestimate due to crossreactivity will naturally be seen only in samples containing plasma.

Another consideration is the speed and convenience of the purification method. Although the charcoal method could be speeded up, at present, with three centrifugation steps, it is scarcely easier than the miniature columns.

In general the LH-20 'minicolumns' are a satisfactory and fairly convenient form of purification of the plasma extracts, especially as expensive columns and fraction collectors are unnecessary.

2.4.3 Incubation with antiserum

Preparation and Storage of Reagents

Buffer: Although the National Institutes of Health recommend borate buffer for this antiserum, phosphate buffer would be more convenient, and so the two buffers were compared.

0.1 M phosphate buffer (buffer p) pH 7.0 was prepared containing 0.1% gelatin. Borate buffer, (B) pH 8.0, was prepared by dissolving boric acid in 250 ml water with 1.35 gm sodium hydroxide. 15 ml conc. HCl was added and the solution made up to 500 ml with water. The pH was adjusted to 8.0 with drops of conc. HCl. To 100 ml of this solution B was added 0.24 gm BSA to give a borate buffer with 0.24% BSA(buffer BBSA).

Three standard curves were prepared and incubated with the three buffers with conditions described in section 2.5.1. The DCC solutions were identical except that they were prepared in the three buffers.

The standard curves are shown in Fig. 2. 13. The non-specific binding was 1.3% for P, 1.2% for B, and 7.2% for BBSA. The least

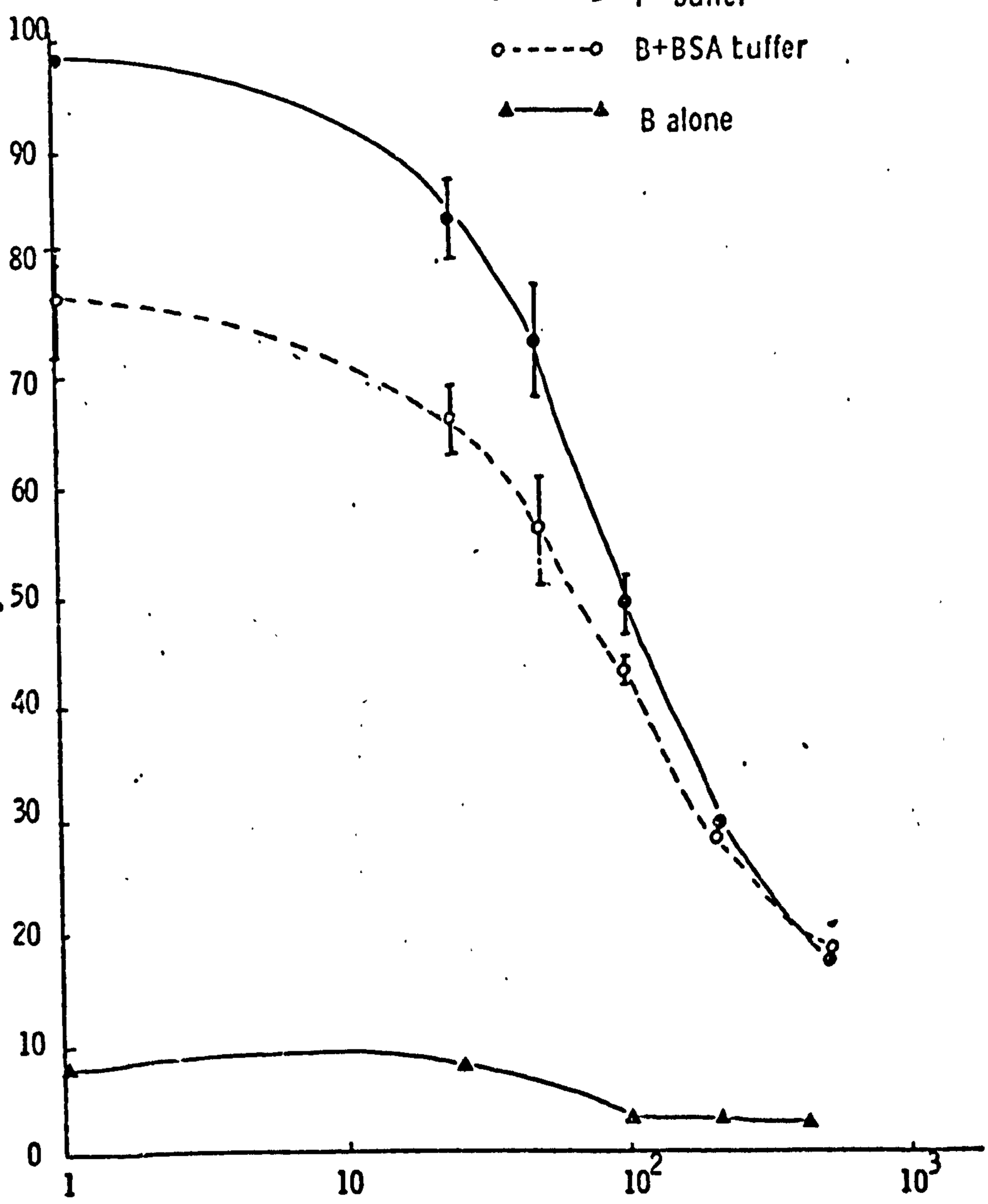


Fig. 2.13 The effect of phosphate (P), borate (B) and borate plus BSA (BBSA) buffers on the standard curve (± S.D, n=3) for aldosterone.

detectable dose was 7 pg for P and 15 pg for BBSA. The omission of BSA from the borate buffer led to an almost complete decline in binding, which could be due to either the ^{3}H-steroid or the antibody coming out of solution.

Phosphate buffer in the incubation led to higher initial binding and a steeper curve, and less non-specific binding than occurs with the buffer containing BSA. Although borate buffer, pH 8.0,with BSA is mentioned in most published methods it seems that the phosphate buffer with gelatin provides a better standard curve and is also cheaper.

Labelled Aldosterone: (1,2-^{3}H) aldosterone was purified as described in 1.3.1. and the purified solution was kept in ethanol. A working solution of 80,000 dpm ^{3}H-aldosterone/ml in pH 7.0 phosphate buffer was prepared at weekly intervals (250 pg/ml).

Standards & Unknowns: The standard stock solution (see Section 1.3.2) was diluted in ethanol to give solutions containing 5, 10, 25, 50, 100, 250, 500 and 1000 pg/0.1 ml, and these were kept for up to two weeks at $4^{o}C$ in glass stoppered tubes. For the standard curve triplicate 0.1 ml volumes of the above dilutions were added to 10 x 75 mm glass assay tubes.

The eluates from the columns were evaporated to dryness at $4^{o}C$ in a stream of air, using the manifold described in the method for progesterone. They were redissolved in 0.35 ml ethanol. Two 0.1 ml volumes were added to assay tubes and 0.1 ml was also added to insert vials to estimate the recovery.

The standards and unknowns were evaporated to dryness in a vacuum oven before the addition of the antiserum and label.

Antiserum: The 1/100 dilution of antiserum was further diluted to give a working solution of 1/250000. 0.25 ml of this was added to

each assay tube.

Incubation Procedure

The effect of the sequence in which reagents are added:

Four standard curves were set up. The antibody working solution (see above) was mixed with an equal volume of the working solution of ^{3}H-aldosterone, and 0.5 ml of this mixture was added to two standard curves, and incubated for 1 hour and 24 hours at 4°C. To the two other standard curves the antibody solution (0.25 ml) was added separately first, and 0.25 ml of the ^{3}H solution was added after 10 mins. Incubations of 1 hour and 24 hours at 4°C were followed by the addition of DCC as described below.

The data are rather scanty but the standard curves are shown in Fig. 2.14. There is a steeper curve at 1 hour if the unlabelled steroid and antibody are preincubated, but this difference disappears at 24 hours, when presumably equilibrium occurs. Rodbard et al (1971) predict that a preincubation will make the assay more sensitive under non-equilibrium conditions, and although this was not proved conclusively on this occasion, a preincubation step was included in further aldosterone assays.

The effect of temperature

Some standard curves were incubated for 5 min. at 37°C before further incubation at 4°C for 1 hour and 3 hours. When these curves were compared with those in which no warming occurred, there was no significant difference between the initial binding (B_0) and the detection limits of the curves.

2.4.4. Separation of antibody-bound and free steroid

Dextran coated charcoal is the preferred separation medium for aldosterone, although the DCC concentrations and volumes differ,

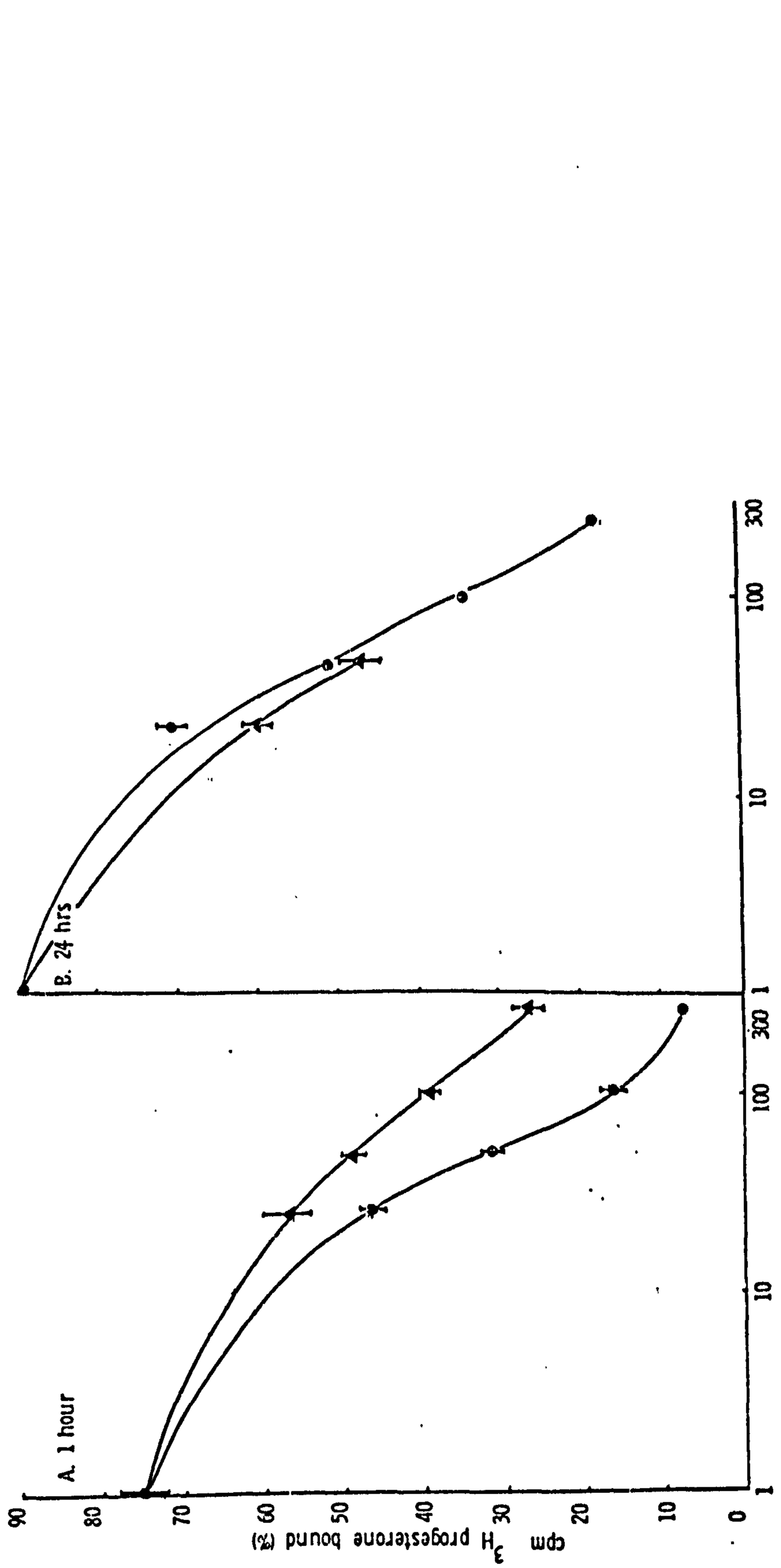


Fig. 2.14 Standard curve (± S.D.) for aldosterone after preincubation of antibody with unlabelled aldosterone (●) or with ^{3}H-labelled aldosterone, (▲) at incubations of (A) 1 hour, or (B) 24 hours.

even for the same antiserum (Waldhaüsl et al., 1972; Vetter et al., 1973). Other methods of separation have been used, such as Florisil (Ito et al., 1972). A novel method has been described by Jowett, Slater, Piyasena & Ekins (1973), in which they extract the free aldosterone directly into a toluene based scintillant. Although this method was reliable, careful control of the timing of the extractions is needed, and another problem is that each separation would require an extraction tube with stopper, which in an average assay would be more than a hundred tubes. Taking these points into account, the toluene based method was not attempted, and the DCC solution from the progesterone assay was tried first.

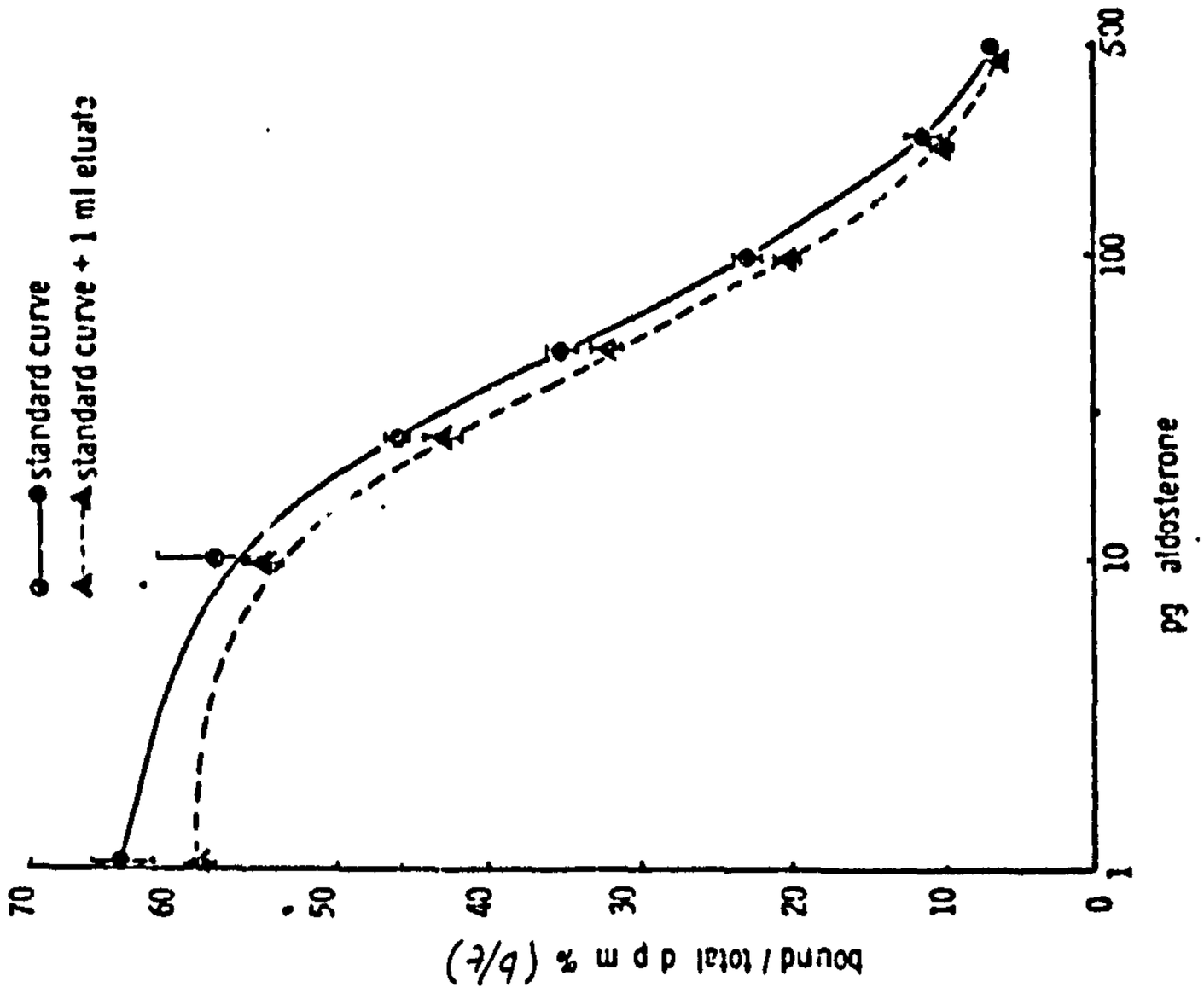
Effect of incubation time

A 'bound' solution of ^{3}H-aldosterone with 1000 pg of unlabelled steroid and antibody was prepared as described above, and a 'free' solution of ^{3}H-aldosterone alone was also prepared. After incubation for 1 hour at 4^{o}C, 0.2 ml of 0.625% charcoal (Norit A), 0.00625% dextran in buffer was added to all the tubes. After various incubation times at 4^{o}C the tubes were centrifuged at 1200 g for 10 min. at 4^{o}C. 0.5 ml of the supernatant was removed and added to insert vials for liquid scintillation counting.

In the antibody blank more than 99% of the counts are adsorbed by the DCC after 5 minutes. When bound counts are present equilibrium is reached by 10 minutes, and there is no increase in counts after this, indicating that 'stripping' of the steroid from the antibody does not occur. This is perhaps a reflection of the high avidity of this antiserum.

2.4.5 Investigation of blank effects

Negative blanks were not a problem in this assay, although positive water blanks did occur sometimes (see 2.5.2). The origin of the positive blank could be either the extracting solvent or the



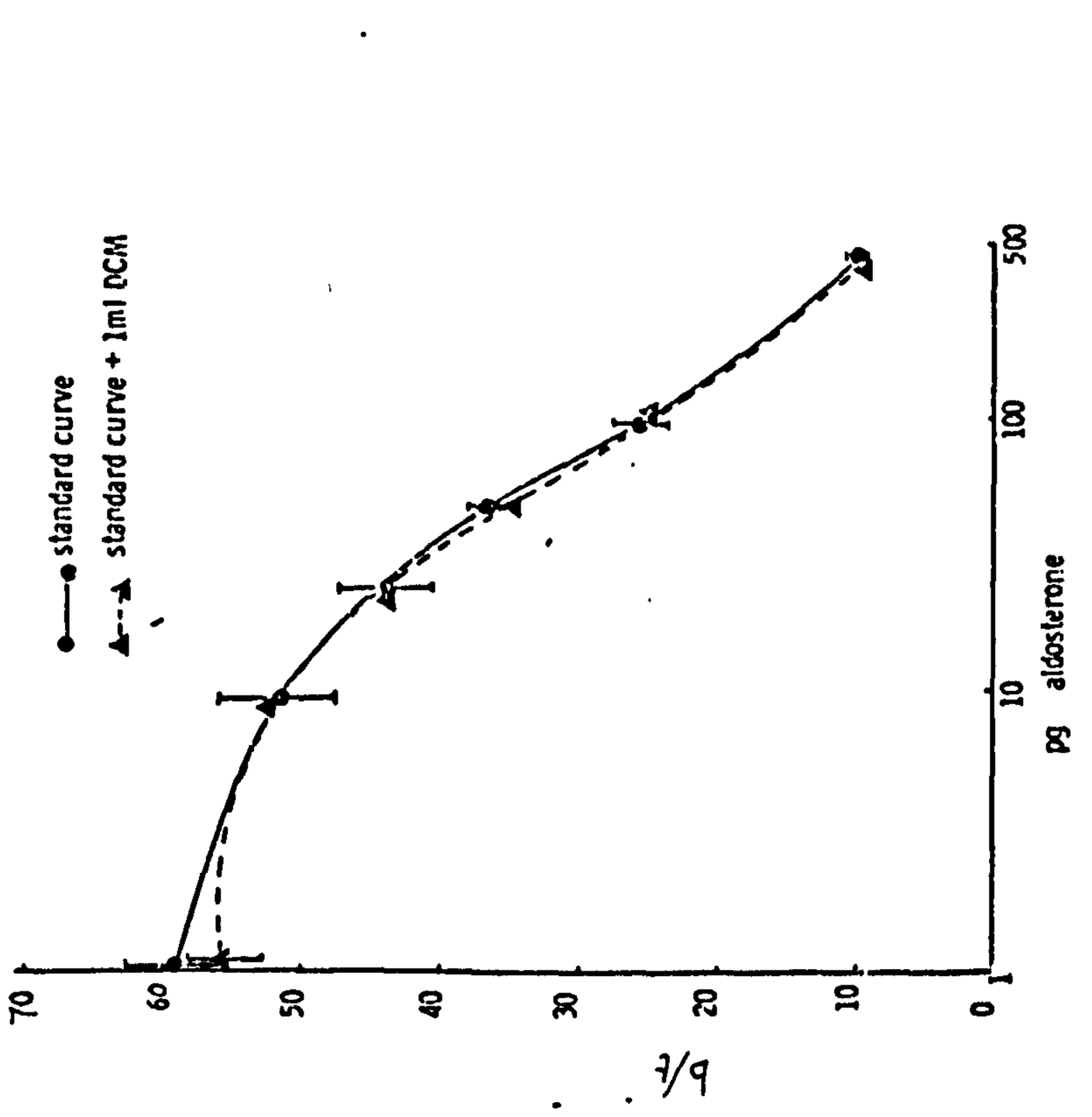


Fig. 2.15 The effect of 1 ml of dichloromethane and of 1 ml of Sephadex LH-20 column eluate (dichloromethane/methanol, 98/2) on the standard curve (± S.D., n = 3) for aldosterone.

chromatography step, and both these were investigated.

The assay procedure is described in earlier sections. In the first experiment 1 ml of DCM (not redistilled) was added to the standard curve, evaporated to dryness, and the standards assayed. In the second experiment 1 ml of eluate from a fresh Sephadex LH-20 column was added to the standard curve and dried.

In the first experiment the DCM residue had no effect on the standard curve (see Fig. 2.15). Although there was a slight fall in binding at the point B_0 this was not significant. In the second experiment there was a significant fall in binding at 0, 25, 50 and 100 pg (see Fig. 2.15). On the basis of these findings it was decided to add 1 ml of column eluate to the assay standards, a procedure also adopted by Ito et al. (1972).

2.5 The Final Aldosterone assay

2.5.1. Summary of procedure

The assay procedure for aldosterone is summarised below.

Extraction: 15 ml glass stoppered tubes were prerinsed with DCM. One ml of plasma was mixed with 5000 dpm (16 pg) of (1,2- ^{3}H) aldosterone in the tubes and left for 30 min. at 20°C.

10 ml of DCM (redistilled) was added and the tubes were vortexed for 30 seconds. The supernatant was removed, using a glass pipette attached to a vacuum line. The dichloromethane was evaporated to dryness in air at 50°C and the extracts redissolved in 0.3 ml of DCM/Methanol, 98/2, prior to chromatography.

Columns: Columns 6.5 cm in height were prepared in prerinsed Pasteur pipettes, using a 3 mm glass bead as a support. The Sephadex LH-20 was soaked for 4 hours in DCM/Methanol, 98/2, and the resulting

slurry was packed into the columns, using a glass fibre disc (cork borer size 3 in diameter) at the top of the columns. Fractions were collected by adding 0.5 ml volumes of solvent mixture to the drained columns (using a Boehringer 'R' pipettor), allowing all the solvent to run through, and transferring the column to a fresh collecting tube before adding the next volume. The first 2 ml were discarded, and the next 1.5 ml collected. This fraction was evaporated to dryness at 50°C in air. 0.33 ml of ethanol was added and the tubes vortexed, then 0.1 ml was taken for recovery and two 0.1 ml fractions were taken for assay. The columns were rinsed through with at least 10 ml of solvent, and kept in a screw-capped jar containing solvent at 4°C, to be used again.

Assay: Standards in 0.1 ml ethanol of 0,5,10,25,50,100,250 and 500 pg and unknowns were added to 10 x 75 mm glass tubes and evaporated to dryness at 40°C in a vacuum oven. A dilution of 1/250,000 of antiserum (no. 088) was prepared in 0.1 M phosphate buffer, pH 7.0 with 0.1% gelatin. A solution of (1,2-^{3}H) aldosterone containing 15,000 dpm/0.25 ml was prepared. 0.25 ml of the antiserum was added to the tubes in random order, and after 10 min. at 4°C 0.25 ml(48 pg) of labelled aldosterone was added to the tubes in the same order. The tubes were mixed gently and left for 1 h at 4°C. 0.2 ml of 0.625% charcoal, 0.00625% dextran in buffer was added to the tubes and after 15 min. at 4°C they were centrifuged for 10 min. at 1200 g at 4°C. 0.5 ml of the supernatant was transferred to an insert counting vial and 3.5 ml of Triton/toluene scintillant added. The vials were counted for 10 min. or 10,000 counts and the assay values were calculated as described in Section 3.

2.5.2. Assay criteria

Standard Curve: The mean B_0 value for 21 assays was 55.3% ± 7.9% and the mean B_{50} value was 75 pg. The mean non-specific

binding was 2 ± 1.5% of the total counts.

Blank: The blank was in the range of 0-5 pg in 18 out of 21 curves, with blanks of 5 to 10 pg on 3 occasions. The mean blank was 2 ± 3 pg. The values found in methods using LH-20 chromatography are generally within twice the standard deviation of B_0, i.e. from 0 to 6 pg (Ito et al., 1972). The blank may still be appreciable in non-chromatographic methods owing to factors in the methylene chloride (Poulsen et al., 1974). McKenzie & Clements (1974) avoided the problem by taking the standard curve through the extraction procedure.

Sensitivity: The detection limit of the standard curve was 3 pg and the corresponding plasma value is 17 pg/ml aldosterone when 1 ml of plasma is taken. Detection limits using the same antiserum as our own (088) have varied from 6 pg (Ito et al., 1972) to 15 pg (Nowaczynski et al., 1974), although values as low as 1.7 pg (Jowett et al., 1973) have been recorded for other antisera. The equivalent sensitivity for a double isotope derivative assay is 140 pg/20 ml (Nowaczynski et al., 1967).

Precision: The interassay precision was tested by means of a quality control sample and the coefficient of variation was 11% (n = 16) for a value of 249 pg/ml. The value found by Ito et al (1972) is 11% and the precision of Daniel et al. (1974) varied from 3 to 14%, with both groups using similar assays to our own. However a C.V. of 22% has been reported for low plasma levels even when chromatography is omitted (McKenzie & Clements, 1974).

The intraassay variability was calculated as described for progesterone. The coefficient of variation for values of 0-100 pg, 100-250 pg/ml and 250-500 pg/ml were 10.1% (n = 16), 10.6% (n = 45) and 8.2% (n = 17) respectively. These are close to the figure of 9.2%

found by McKenzie & Clements (1974).

Accuracy: 50 to 1000 pg of aldosterone was added to water and assayed. The values obtained can be represented by the equation:

$y = 0.998x - .73 \quad r = 0.998 \quad (n = 41)$

An accuracy study was not carried out in plasma, but the results in water shown no apparent systematic error. An accuracy study was carried out with adrenalectomised plasma using the same antiserum as our own after LH - 20 columns, and the accuracy was 102 ± 2.6% (Ito et al., 1972)

Recovery: The recovery of ^{3}H-aldosterone added to plasma was 58 ± 9% in 18 assays. Recoveries in this range are found for paper chromatography (Migeon et al., 1973) and LH - 20 chromatography (Ito et al., 1973) but recoveries after silica gel column chromatography are slightly higher at 77 ± 5% (Mayes et al., 1970). Recoveries after solvent extraction alone may be 85 - 90% (McKenzie & Clements, 1974; Poulsen et al., 1974).

Conclusions:

Although this method has now been superseded by methods using more specific antisera, the reliability criteria are comparable with these newer methods in all respects apart from the sensitivity and speed.

2.6 OESTRADIOL

An assay for 17β-oestradiol was developed by other members of this Department, particularly by Mr S Ord.

2.6.1. Antiserum

The antisera (R3 & R4)kindly provided by Dr. P.D.G. Dean, were raised against 17β-oestradiol-6(-o-carboxymethyl)-oxime BSA in rabbits.

Titre: The titre of the first batch, R4, was found to be 1/5000 when 0.1 ml of this solution was added to the assay. The titre of the second batch, R3, was 1/15,000 under similar conditions.

Specificity: The cross-reactivity of the antisera are described by Exley, Johnson & Dean (1971). The cross-reactions with oestriol and œstrone are in the order of 1%, and very low cross-reactions (0.01%) are evident for non-oestrogenic steroids. Other oestrogens such as oestrone are present to an equal or lesser extent than oestradiol in menstrual cycles, so that cross-reactions of 1% or so should not create problems.

Affinity Constant: The affinity constant was calculated from a Scatchard curve and was found to be 2×10^{10} l/mol.

2.6.2. Summary of Procedure

Thanks to the highly specific antisera, described above, the assay did not require purification of the plasma beyond a simple extraction to remove non-specific factors. The procedure is outlined below; details of the reagents used will be found in preceding sections.

Extraction

The solvent most commonly used for the extraction of œstradiol is diethyl ether (Abraham, 1969; Mikhail, Wu, Ferin & Vande Wiele, 1970), although Dr. P. Hurt in this Department found that extraction

was as good with dichloromethane. Dichloromethane is non-flammable, yet with a low boiling point for rapid evaporation, and it was chosen for the extractions until assay No. 15. However after this the accuracy in the next few assays was very low, and it was found that diethyl ether extractions were preferable, so that ether was used in assays after this.

15 ml glass stoppered tubes were rinsed with 1 ml of the solvent to be used, and 2000 dpm (3 pg) of ^{3}H - labelled 17β-oestradiol was added to each tube, together with 0.5 - 1.0 ml. of the plasma samples, in duplicate. The mixture was left for 1 h at 20°C.

If dichloromethane was used; 10 ml of redistilled dichloromethane was added, the tubes vortexed for one min. and the aqueous layer removed by suction. The solvent was evaporated to dryness at 50°C in air.

If diethyl ether was used; 5 ml. of ether was added to the tubes and vortexed for one min. The bottom layer was frozen in a mixture of solid carbon dioxide chips in ethanol, and the ether layer was decanted and evaporated to dryness at 40°C in air.

The residues from both extractions were redissolved overnight in 0.35 ml. buffer and vortexed the next day. Two 0.1 volumes were added to assay tubes and 0.1 ml. was added to scintillation vials to determine recovery.

Assay

A solution of ^{3}H-labelled 17β -oestradiol of 150,000 dpm/ml in 0.1 M phosphate buffer was prepared weekly from the repurified stock solution. Standard solutions of 17β -oestradiol were prepared weekly in buffer from the stock solution of 1 mg/ml in ethanol, and volumes of 0.1 ml of buffer containing 0,2.5, 5, 10, 25, 50, 100 and

250 pg of 17β-oestradiol were added in triplicate to assay tubes. 0.1 ml of the antiserum (see above) was added to the standards and unknowns and after an incubation for 30 min at 4°C, 0.1 ml (20 pg) of the label solution was added, the tubes were incubated at 4°C for a further hour.

0.5 ml of 0.5% charcoal, 0.05% dextran in buffer was added to all the tubes, vortexed briefly, and incubated at 4°C for 20 min. The tubes were centrifuged at 1200g for 10 min. at 4°C. 0.5 ml. of the supernatant was removed, using a Selectapette, and placed into insert vials for liquid scintillation counting, as described in Section 1.2.

2.6.3. Assay Criteria

Standard Curve

The mean B_0 for R4 was 72 ± 8% (n = 8) and R3 was 69 ± 6% (n = 2). The detection limits of the curve were 3 pg for R4 and 1.5 pg for the other antiserum, R3. Kushinsky & Anderson (1974) reported a detection limit of 1 pg, but other quoted limits have been as high as 10 pg (Powell & Stevens 1973). High sensitivity for œstradiol is important as the plasma levels are especially low in most circumstances. The B_{50} values were 50 pg for R4 and 22 pg for R3. The mean non-specific binding was 3 ± 1.8% for R4 and 7.5 ± 2% for R3.

Blanks: The water blank was within the standard deviation of the zero point in all but 3 assays. Two assays had 'negative blank' and in one assay there was a positive blank of 25 pg. This assay was discarded. The mean positive blank was 1.5 ± 1 pg (n = 10).

In earlier column chromatographic methods, the blank could be 8 ± 6 pg (Emment et al., 1972), or even 11 pg (Mikhail et al.,

1970), which meant volumes of plasma of up to 5 ml had to be used to overcome this effect. In non-chromatographic assays England et al., (1974), using benzene to extract, found a blank of 4 pg, while Aso et al., (1975), using ether, found a value of 0.2 pg.

Sensitivity: The detection limit was taken as 3 pg, which meant that if 1 ml of plasma was assayed and 20% of this extracted, then the sensitivity was 15 pg/ml plasma.

Precision: The intra-assay precision of the sample measured in duplicate, for the values 10-100, 100 - 200, and 200 pg/ml, were 9.9% (n = 31), 11.0% (n = 43) and 9.1% (n = 20) respectively. Aso et al (1975) found the mean coefficient of variation for a whole range of duplicates was 7.4% but values of up to 19% have been recorded, even when the extraction step is omitted (Jurjens et al., 1975).

The inter-assay precision (quality control) was estimated from two plasma pools to be 10% (n = 4) for a mean of 88 pg/ml and 17% (n = 5) for a mean of 120 pg/ml. England et al. (1974) obtained an inter-assay precision of 6 - 7%, while the corresponding figure for Aso et al. (1975) was 18% (n = 16).

Accuracy: The accuracy was tested by adding standard amounts of oestradiol to water blanks. The recovery of 100 pg was 118% (n = 2) and the recovery of 250 pg was 94% (n = 8). The accuracy was also tested by assaying four dilutions of the same plasma pool. The regression coefficient was 0.99 and the results obtained are described by the equation;

(observed) = 1.10 (expected) - 12.4 pg/ml.

An exchange of samples was carried out with the Chemical Pathology Department and the following equation was obtained:

(Gynae. Dept. results) = 0.84 (Chem. Path. results) + 9.4 r = 0.99

Recovery: The recovery of ^{3}H-oestradiol added to plasma before assay was 70 ± 11% (n = 60) when dichloromethane was used and 74 ± 9% (n = 48) with diethyl ether. These results are rather low compared to 97% found by Aso et al. (1975) after a single ether extraction. These authors do however vortex mix the extracts for at least one minute, which is twice as long as the procedure usual in this department.

Conclusions

The oestradiol assay was difficult to run in that periodically the assay broke down, and the standard curve or accuracy went wrong for no apparent reason. However the assay finally stabilised and the assay criteria are comparable with other authors.

2.7 Prolactin

Prolactin determinations were carried out by Dr. M.J. Wheeler, Department of Chemical Pathology and Metabolic Disorders, St. Thomas' Hospital, using a radioimmunoassay.

Antiserum

Antiserum, batch 7110, was supplied by Dr. G. Groom, Tenovus Institute, Cardiff. Although it was recommended that this antiserum was used at a working dilution of 1/6000, it was found that the assay protocol employed allowed a working dilution of 1/10,000, equivalent to a final dilution of 1/70,000. There was no significant cross-reaction with growth hormone (< 1%), although it is unlikely that any of the patients studied would have had significant growth hormone

levels as there were not acromegalic, had not eaten for several hours and were not unduly stressed.

Material for iodination

Highly purified prolactin was obtained free from the National Institutes of Health, Bethesda, Maryland, USA. 2 µg hormone were iodinated either by using the chloramine - T method or the lactoperoxidase method. After iodination, the iodinated hormone was separated from free 125-Iodine by column chromatography, using Sephadex G-100 in a 100 cm column. 1.5 ml aliquots were collected with the iodinated hormone being eluted in fractions 47-53. The middle fractions of the prolactin peak, fractions 49 -51, were bulked, aliquoted as 500 µl amounts and stored at -20°C. No further purification was carried out before use. When fresh this preparation gave a zero binding of 45%, dropping to 25% over six weeks but without any loss in assay performance as determined by the assay quality control samples.

Radioimmunoassay

50 µl standard or sample were added with 450 µl 2.5% horse serum in phosphate-buffered saline, pH 7.2, to 75 cmx 12 cm polystyrene tubes (RT30, Sterilin Ltd) using an LKB Autodilutor. 100 µl antiserum was added, the tubes mixed and the assay stored at 4°C. Zero tubes containing no standard and blank tubes containing no antiserum were prepared at the same time.

After 18-24 hours incubation 100 µl prolactin, equivalent to between 10,000 and 15,000 cpm was added to each tubes, with 100 µl being added to two empty tubes - the total count tubes.

After a further incubation of 18-24 hours at 4°C, 100 µl of a solution of rabbit serum and donkey anti-rabbit gammaglobulin, at dilutions of 1/20 and 1/200 respectively was added to all tubes except the total tubes.

The tubes were incubated for at least a further 18 hours, centrifuged at 3,000 rpm and the supernatant aspirated. The amount of radioactivity in the precipitate remaining, equivalent to the anti-body bound labelled hormone was determined using a Gammaset 500 (Tracerlab Instruments Ltd). Unknowns were read from the standard curve subsequently produced.

Assay Performance

The routine assay used had a sensitivity, determined as the 10% drop from the zero binding, of about 100 miu/l (using MRC standard 71/222). This meant that the lowest values of the normal population could not be measured and therefore the normal range is quoted as < 480 miu/l.

The intra-assay variability is 5.4% with an interassay variability of 9.3%.

3. ANALYSIS OF DATA

General Statistics

In the calculations the mean ($\bar{x}$) and standard deviation (SD) are usually given as ($\bar{x} \pm SD$) followed by (n), the number of observations. In some cases the standard error (SE) of the mean is given; this is defined as $\frac{SD}{\sqrt{n}}$. The coefficient of variation (CV) is $\frac{SD}{\bar{x}} .100\%$

It has been shown (Kletzky, Nakamura, Thorneycroft & Mishell, 1975) that progesterone and oestradiol in the menstrual cycle tend to follow a logarithmic normal rather than a normal distribution. Accordingly steroid values were transformed logarithmically and the grouped values calculated as the log mean and log standard deviation. However the antilog of the standard deviation cannot

be derived directly but is calculated as (antilog. mean - antilog (log. mean- log. SD))for the SD below the mean and (antilog. (log. mean + log. SD) -antilog. mean)) for the SD above the mean. As these two values are not equal, the mean and standard deviations of plasma values are expressed as $\bar{x}$ (SD_u, SD_1) where SD_u is the upper standard deviation and SD_1 is the lower one.

Statistical treatment of the normalised data was, unless otherwise stated, comparison of unpaired means (Students 't' test) and linear regressions using the Compucorp 344 Statistician.

Assay Calculations

The plotting of the standard curve for all the assays was the B/B_0 or the B/T vs log. dose, as described on page 79.

Standards were assayed in triplicate and 'unknown' plasma extracts in duplicate. The mean dpm value for each standard or unknown was calculated and the standard curve was plotted as (dpm / dpm at B_0) x 100% vs. log. pg standard. Values for unknowns were interpolated on the curve and the final result calculated as follows:-

$$X = \frac{100 \cdot x}{r \cdot V}$$

X - value in pg / ml plasma

x - value interpolated from S.C of 0.1 ml extract (pg)

r - percentage recovery of I.S. in 0.1 ml extract

V - volume plasma assayed (ml)

Values of pg/ml or ng/ml of plasma were then converted to S.I. units of nmol/l.

Arranging the plasma values

One of the problems associated with the collection of data in the menstrual cycle is the adjustment of different cycle lengths. This can be done on an absolute basis, counting from

ovulation as the reference point. Ideally the day of the LH peak should be used, but as daily LH determinations were not available in our study the day of the rise in body basal temperature (BBT) was the reference point. However the problems of determining the exact day of ovulation from the BBT are well known.

As the second half of the cycle is less variable than the first (Marshall, 1963) it would seem reasonable also to use the onset of the next menses as the reference point. Accordingly the data were arranged both ways, around the onset of the next period and also around the day of the first increase in body basal temperature where this was known (for instance, in Group I, see page 144).

The computer model

The cycle can also be divided into proportional units for comparison between different women (Redgrove, 1971). As progesterone values are really only of interest in the luteal phase (the levels are approaching the sensitivity of the assay in the follicular phase) it was decided to attempt such a comparison of luteal progesterone values.

Progesterone values appear to fit a periodic or wave-like shape, and a computer programme was devised by Dr. S.C. Darby using a sine wave model. The luteal phase was divided into proportional units (radians) and the corresponding progesterone values fitted to the sine wave programme, which calculates the goodness-of-fit of the sine curve and the maximum progesterone values at the mid-point in the luteal phase. This maximum amplitude of the sine wave is referred to as the progesterone 'index'.

Validation of the computer model

One problem with determining a progesterone index by the computer model is that the luteal phase length should be known.

As this information was not available for all of the women it was decided to take an arbitrary luteal phase length of 14 days where the true figure was not known. The effect of such an approximation was tested by calculating the indices for the first group (see Ch.3) in both ways; with the true (known) luteal phase and with an arbitrary luteal phase. The results are shown in Table 2.5.

Table 2.5 Computer indices of progesterone secretion calculated using either the true luteal phase length, or an arbitrary length of 14 days.

	Actual luteal phase		Arbitrary luteal phase	
	Index nmol/l	S.D.	Index nmol/l	S.D.
Controls (10)	48.7	7.7	43.1	7.4
PMS group 1 (20)	37.3	8.7	36.8	9.4
	$p < 0.05$		n.s.	

Apparently therefore, using an arbitrary luteal phase may give anomalous results and may obscure differences between groups.

One test of the model is to compare the index values obtained by computer with the estimates of 'low' and 'normal' progesterone obtained by comparing each progesterone curve graphically with the 90% confidence limits of the controls (see Ch. 4). In Fig. 2.16 it appears that the computer model, obtained via an arbitrary luteal phase length, is a good discriminator between normal and low progesterone curves.

Another test of the model was to compare the computer 'peak' (y) with the true progesterone peak (x) obtained on day 7 before menstruation, where this was available. The correlation between the two is described by the equation:

$$y = 13.8 + 0.6x, \quad r = 0.83 \quad (n = 39)$$

It appears therefore that the computer model tends to under-

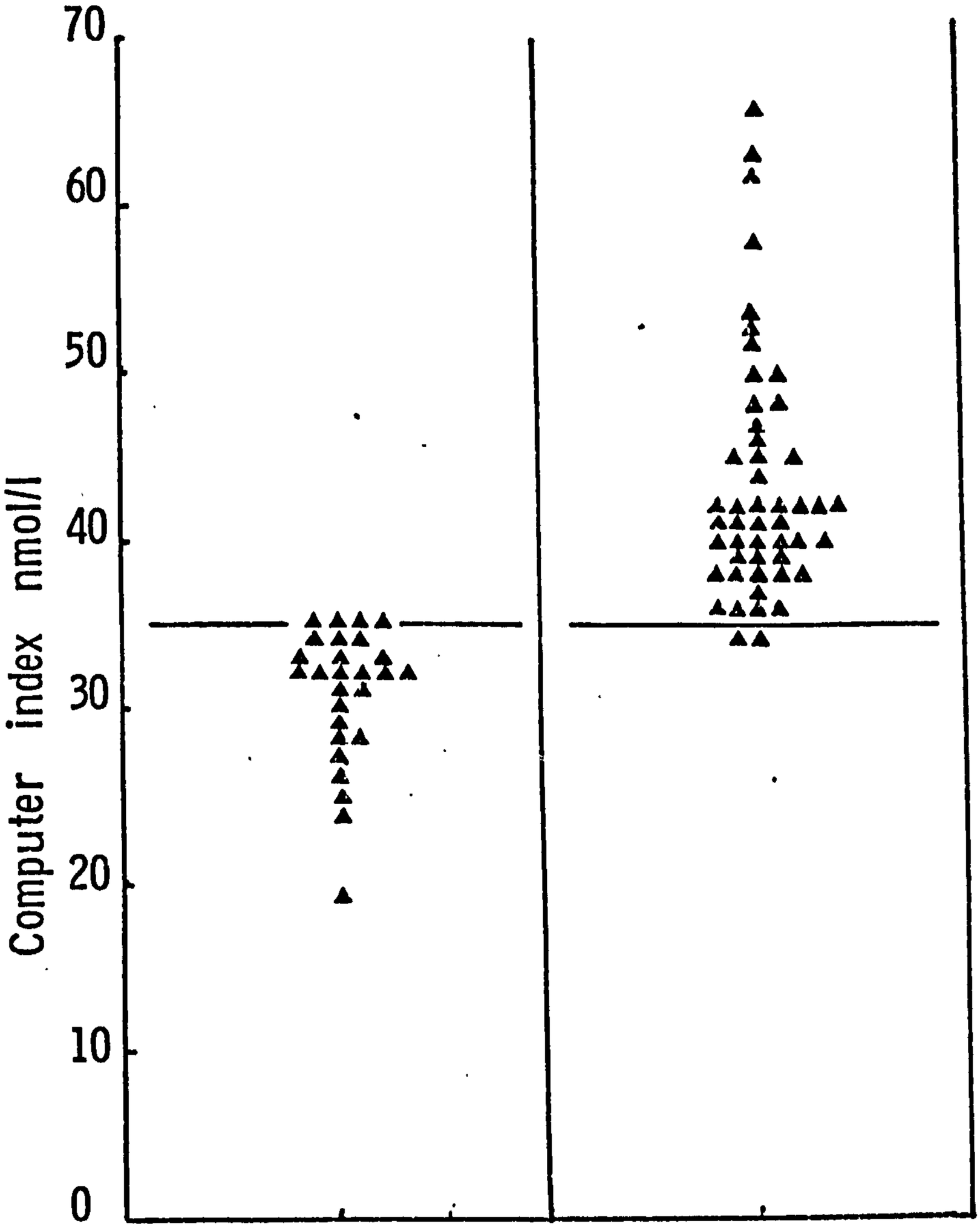


Fig. 2.16 Test of the computer model of the progesterone peak; the progesterone index obtained by the computer programme for each individual is compared with an independent, graphical classification of each patients progesterone curve, whether 'normal' or 'low' (below 90% confidence limits of a control group).

estimate high peak values and to overestimate low ones. This is not surprising in view of the flattened shape of the sine wave peak, and it may also be a consequence of the logarithmic distribution of progesterone values (see above) which is not taken into account by the computer model.

However it may be an advantage since an integrated value may be a better indicator of luteal function; Abraham et al., (1974) have shown that one high value may be misleading with regard to luteal function. Computer indices were calculated for all women in Group I and III, using the true luteal phase length in each case, but an arbitary luteal phase of 14 days had to be used in the calculation for group II.

4. CLINICAL METHODS

The patients chosen for the study all attended the special research clinic for the premenstrual syndrome, and they were seen by Professor R.W. Taylor or one of his colleagues for clinical assessment. Although some patients were referred following articles in the press, in every case the G.P was informed and his or her consent obtained before the study.

Details of the patients and the protocol followed for each study (for instance the timing of blood samples) are given in the appropriate Results Chapters.

Blood Samples

20 ml of blood (usually for progesterone and oestradiol determination) was taken from the antecubital vein between 9.30 am and 15.30 pm, placed in heparinised tubes and centrifuged as soon as possible at 1000g for 10 min. For prolactin estimations 10 ml blood

was placed in glass tubes, allowed to clot for 30 min at 20°C, and centrifuged as above. All samples were frozen at -20°C before assay.

Research Design

The endocrine investigations were carried out in three parts, governed to some extent by the patients available at each stage.

These studies may be summarised below.

Group I. There was a comparison of symptomatology and menstrual cycle details in 20 women complaining of the premenstrual syndrome and seven women who were not. Plasma levels of progesterone, oestradiol and aldosterone were studied frequently during the cycle. These results are given in Chapter 3.

Group II. Progesterone levels were studied within a heterogenous group of 105 PMS patients, especially with regard to symptoms and psychological criteria. The relationship between prolactin and progesterone was also studied (Chapter 4).

Group III. In 67 PMS patients treated with dydrogesterone in a single-blind trial pre-treatment levels of progesterone were compared with levels during treatment, and both values were correlated with response to the therapy. (Chapter 5).

CHAPTER 3

Plasma steroid profiles in women with premenstrual syndrome and in controls.

1. SUBJECTS

Patient Protocol

Seven volunteers aged 22 to 34, without premenstrual symptoms were recruited from the hospital staff and were studied over ten cycles, with three women contributing two cycles each. These ten cycles acted as the controls.

Twenty women with premenstrual syndrome (Group I) were selected for the investigation. Seven of these were referred from the Gynaecology Out-Patients clinic, while the otherswere self-referred following a magazine article which called for volunteers. Although the latter patients were chosen from a much larger group of volunteers (see Chapter 4), they were chosen in chronological order of their appearance at the clinic, so that bias in selection should not occur.

All the women fulfilled the following criteria:-

1) The women were aged 22 to 45, with regular cycles, and they were taking no medications, apart from simple painkillers, during the period of study.

2. They were available for at least four blood samples in the luteal phase and these were taken between February 1975 and August 1976, so that plasma samples from both PMS and control groups were assayed in the same batches.

3. The subjects had a complete daily record of basal body temperature (BBT), weight and symptoms during the cycle under study.

4. In the case of the women with PMS, it was established that the symptoms were cyclical and that they ended at or soon after the onset of menses.

Thus the details of ovulation (by BBT), weight and symptoms were available together with progesterone and oestradiol analyses during the luteal phase.

All the controls and a subgroup of the PMS series (the first eight) had blood taken for aldosterone estimations. Most studies of aldosterone in the menstrual cycle have been carried out on patients who have been supine overnight (Michelakis et al., 1975; Katz & Romfh, 1972) but overnight admissions were not possible for our study due to the shortage of hospital beds. In other out-patient studies, for instance in pregnancy, subjects remain recumbent for 30 minutes (Weir, Brown, Fraser, Lever, Logan, McIlwaine, Morton, Robertson & Tree, 1975). It was decided therefore to adopt a protocol of 30 minutes recumbency with sampling between 10.00 and 15.30 h. To check that recumbency did not alter gonadal steroids, the progesterone index of 'recumbent' and 'non-recumbent' subjects in the PMS group were compared. The mean index of the 'recumbent' group (10) was 30.3±5.1 nmol/l and that of the non-recumbent group (12) was 35.7±4.8 nmol/l, (not significant). No attempt was made to control the diet of these patients.

Details of the menstrual cycle and symptoms

The symptoms experienced by the PMS subjects and the controls after the rise in body basal temperature are shown in Table 3.1. Only symptoms that were rated as 'moderate' or 'severe' or that occurred on more than one day of the cycle were included.

It is interesting from this table that breast tenderness occurred in almost half the control cycles, and that the most striking differences between the groups are in the emotional symptoms, although the ratings are very subjective. It is also interesting that at the original clinic visit the PMS patients had

Table 3.1 The incidence of symptoms during 20 cycles of 20 PMS patients and 10 cycles of 7 controls, taken from symptom diaries.

	PMS Cycles (20)			Control Cycles (10)		
Symptom	No.	Percent	Mean No. days	No.	Percent	Mean No. days
Depression	19	95	5	0	0	-
Irritability	17	85	5	0	0	-
Lethargy	11	55	7	0	0	-
Swollen, tender breasts	13	65	8	4	40	4
Swollen abdomen	14	70	9	1	10	4
Headache	10	50	4	0	0	-
Weight Gain (2lb or more)	6	43*	5	0	0	-
Stomach cramps (premenstrual)	4	25	7	0	0	-
Stomach cramps (menstrual)	9	45	2	2	20	2

* only 14 recorded weight daily

been asked about the duration of their symptoms, and they reported that symptoms occurred from 2 to 19 days, with a mean of 9 days, which is longer than the actual duration of most of the symptoms listed in Table 3.1. This underlines the problems in dealing with retrospective questionnaires.

Some of the symptoms were maintained after menstruation had begun, and an indication of the timing of symptoms in the PMS group is shown in Fig. 3.1, which was obtained by adding the scores for each premenstrual day.

Details of the menstrual cycles during the study are shown in Table 3.2. The length of the luteal phase was calculated from the day of the rise in body basal temperature, and as this method is rather unreliable, the estimation must be regarded as approximate.

Table 3.2. The mean age, cycle length, luteal phase and flow in PMS group and controls.

	PMS group (20)		Controls (10)		P
	Mean ± SD	Range	Mean ± SD	Range	
Age (years)	31 ± 6	22-45	28 ± 3	22-34	ns
Length cycle (days)	27.3 ± 2	25-32	29.4 ± 2	26-32	<0.01
Length luteal phase (days)	13.2 ± 1.4	10-15	13.7 ± 1.0	12-15	ns
Menstrual flow (days)	4.5	2-9	5.2	4-9	ns

There was a slight but non-significant difference in the age distribution of the two groups, although some effort was made to overcome this by selecting younger PMS patients for study. It would have been better to select older controls, to match the age range of

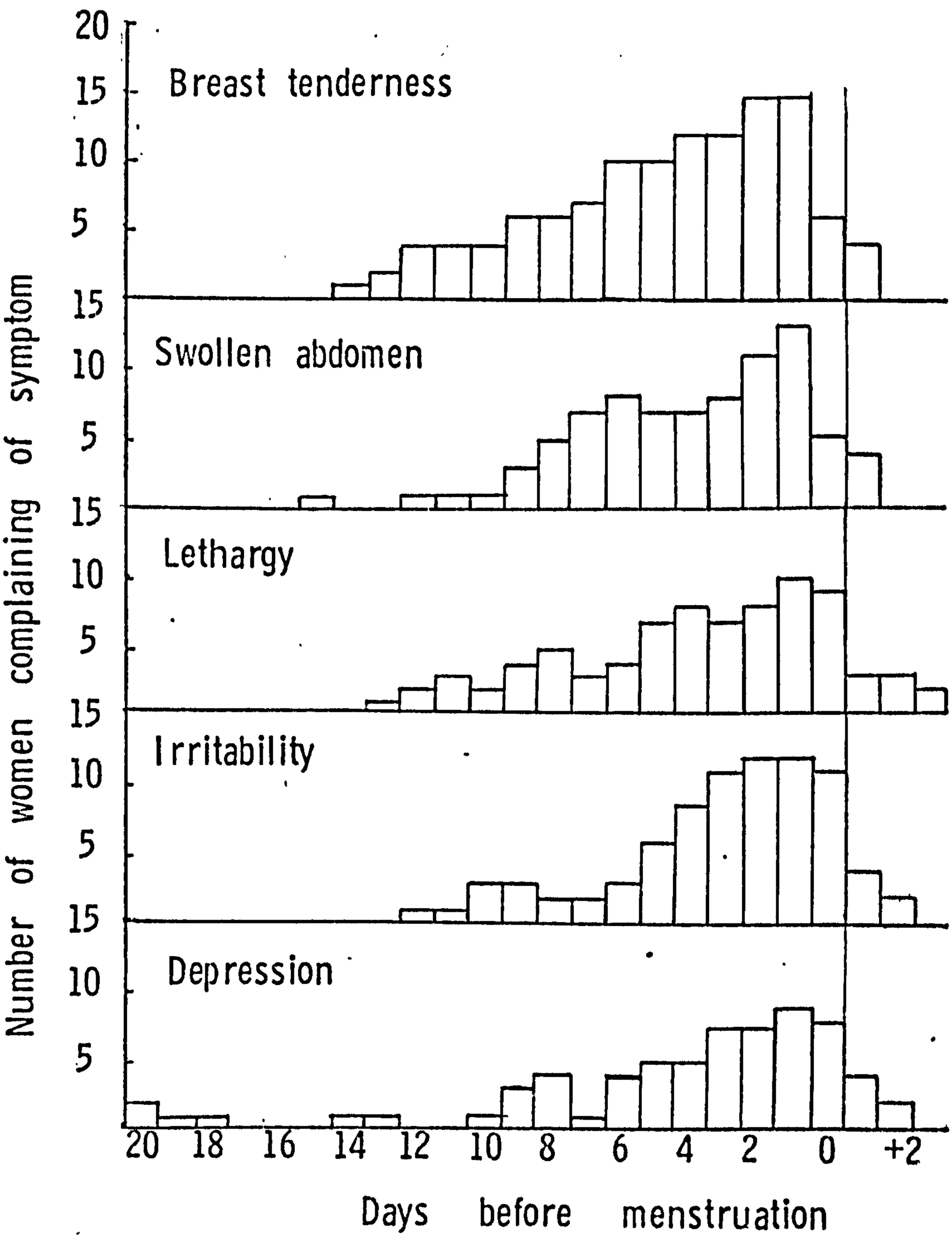


Figure 3.1. The daily pattern of total self-rated symptom scores from 20 women with premenstrual syndrome.

the PMS group, but this proved difficult as the controls were drawn from a population of students, nurses and technicians. There may also be a true age effect since the syndrome is most often seen in women over 30 (Dalton, 1977). Indeed, it was extremely difficult to find subjects in their late twenties and over who were asymptomatic and who were not taking oral contraceptives.

The difference in menstrual cycle length may be an effect of the difference in age distribution, since it has been shown on a statistical basis that cycles become progressively shorter (Treloar et al., 1967; Chiazze et al., 1968). In a study of 3,300 regular cycles Chiazze et al. (1968) found that the average cycle length for women aged 25 to 29 was 28.5 days, which is slightly shorter than the average for our controls. The average cycle for women aged 30 to 34 was 28.0 years, which is slightly longer than that found in our PMS group.

It has been shown by Sherman, West & Korenman (1976) that although cycles tend to become shorter in older women, it is the follicular phase and not the luteal phase that is decreased, and the same phenomenom would appear to occur in Group I, as there was no statistically significant difference in luteal phase length.

2. PLASMA STEROID PROFILES

Progesterone

Controls: The progesterone values obtained in the control patients are compared with values found by other workers in Table 3.3. The follicular phase values are slightly lower than those of most workers, although all reported levels are below 2 nmol/l. The luteal phase values are within the range found by other workers. Although the other papers quoted were not selected for the absence of premenstrual

Table 3.3 Comparison of Plasma Progesterone values obtained by different authors for the menstrual cycle

		Subjects				PLASMA PROGESTERONE, nmol/l mean, ±SD, (range)					
Author	Method	No.	mean Age years	mean Menstrual cycle days	mean luteal phase days	Follicular	day LHO,+1	day M-8 to M-5	Midluteal peak (M-7)	day M-1	day MO
This Study	RIA	10	28 (22-34)	29.4	13.7	0.7±0.3	3.1±1.8	45±14	49.6±21	6.8 (3-12)	0.7
Guerrero et al (1976)	RIA	17	32	28.3	13.4	0.95	3.65	48±5.9	55	5.2	3.1
Florensa et al (1976)	RIA	5	21-28	27-30		1.3±.3	3.8±.6		56.3±17	1.3-5.7	
Israel et al (1972)	RIA	13			13	1.8			48.3		
Johansson (1969)	CPB	20	18-32	29.6±3	14±1	~ 1.6	~3.8	~44		4.0±3.0	1.9±1.0

M = first day menses

LH = day of LH peak

CPB is competitive protein binding assay

RIA is radioimmunoassay

symptoms it can be assumed from the age ranges that severe PMS was uncommon.

However, a more accurate comparison of the control values (for the luteal phase only) can be obtained with the composite graph of Bäckström, et al (1976b)who were also selecting controls for a PMS survey. The comparison in Fig. 3.2., indicates lower values in our control group, although the same pattern is seen. The discrepancy probably reflects methodological differences.

PMS Group

The progesterone levels of the PMS and control group were arranged around the day of the onset of the next period and the log. means were calculated. A comparison of the curves for the PMS and controls are shown in Fig.3.3. The progesterone levels of the PMS group were lower in the midluteal phase, that is, from -10 to -5 days premenstrually. As fairly small numbers of samples were available per day in both the groups, the data was grouped into two day intervals around the onset of menses (Table 3.4), but even with this arrangement the difference in the two groups only reached significance at 7-8 days premenstrually, although the PMS group had lower values from 5-10 days. When the data are further pooled into four day intervals (Table 3.4) the PMS group have significantly lower progesterone levels during the mid-luteal plateau at 5-8 days premenstrually (M-5 to -8). There was no significant difference between the progesterone levels on the last four days of the cycle, and a valid comparison is difficult from day -11 to -14 owing to the different luteal phase lengths, and this is reflected in the high variability at this time.

The values were also arranged around the estimated days of ovulation and the log. means calculated (Fig. 3.4), since more information can be obtained in this way about the levels in relation

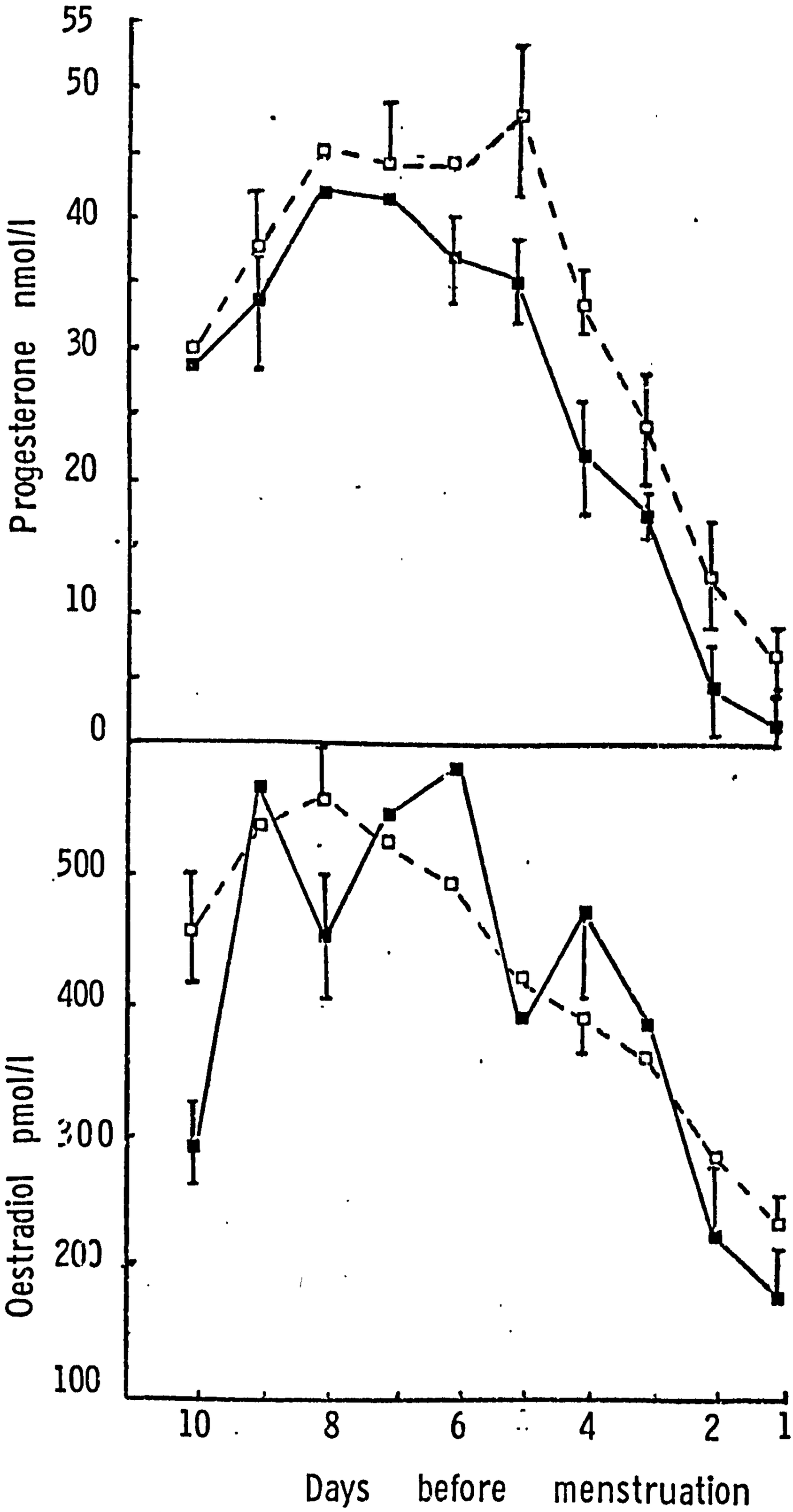


Figure 3.2. Daily mean(± S.E.)plasma progesterone and oestradiol levels in ten control patients in this study (■——□) compared with mean values found for controls by Bäckström et al (1976b)(□- - -□).

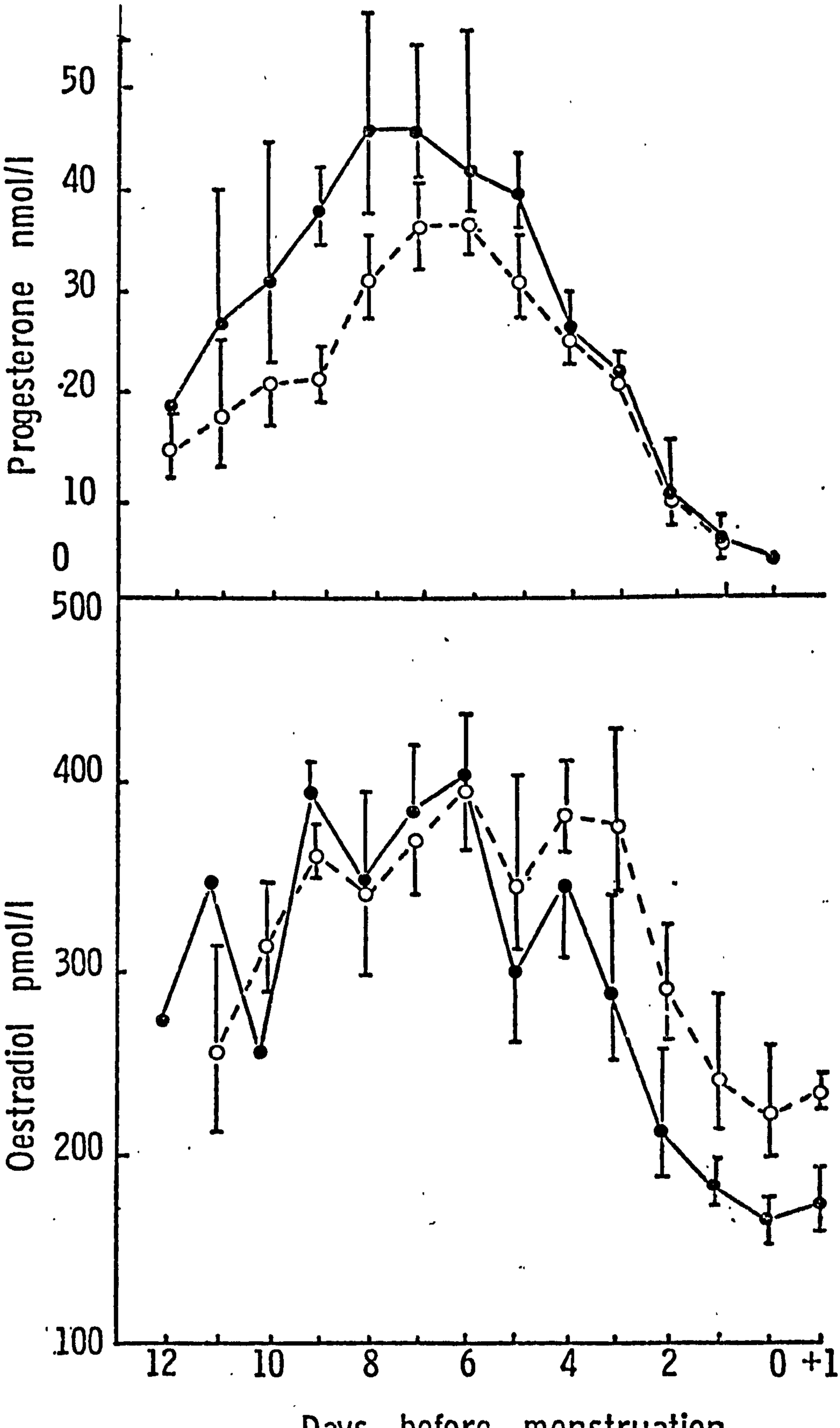


Figure 3.3. Logarithmic mean (± S.E.) plasma progesterone and oestradiol levels prior to menstruation in 20 women with PMS (O- - - -O) and in ten control cycles(O——●).

Table 3.4 Log. Mean Progesterone Values (and Standard Deviations) throughout the Luteal Phase, Dated Backwards from Menstruation (M) in PMS Patients and Controls

Day cycle M-	Controls			PMS Patients			P (probability)
	No. samples	Mean nmol/l	S.D. upper , lower	No. samples	Mean nmol/l	S.D. upper , lower	
MO,+1	6	5.2	1.6 , 1.3	6	5.5	3.8 , 2.5	ns
1 - 2	7	6.0	4.4 , 2.5	7	7.0	6.4 , 3.8	ns
3 - 4	8	23.6	5.4 , 4.4	15	24.1	9.2 , 6.7	ns
5 - 6	8	40.1	10 , 8.3	15	34.0	13 , 9.2	ns
7 - 8	9	45.8	19 , 13	11	33.4	11 , 8.6	<0.05
9 - 10	7	33.4	19 , 12	10	21.0	15 , 8.6	ns
11 - 12	7	16.2	23 , 9.5	6	17.2	16 , 8.3	ns
1 - 4	15	12.7	16 , 7	22	14.6	20 , 8.3	ns
5 - 8	17	39.1	17 , 12	26	29.6	16 , 10	<0.02
9 - 12	14	27.1	28 , 14	16	19.2	14 , 8.3	ns

to ovulation itself. In Fig. 3.4, the initial rise in progesterone after ovulation seems unimpaired in the PMS group, although secretion seems to decrease 5 days after ovulation, so that the luteal peak is smaller and the fall in levels occurs slightly earlier. Menses occur in both groups when levels of progesterone reach about 4 nmol/l.

Oestradiol

Controls: The oestradiol plasma values of the controls were compared with other workers (Table 3.5) and found to show good agreement with values for the menstrual cycle obtained by both RIA and CPB. The midcycle peak is rather low but very few samples were obtained at that time in our study.

The oestradiol levels in the controls showed the characteristic pattern of low follicular phase levels, a sharp preovulatory peak and a nadir at ovulation. The luteal phase levels rose again to a plateau which was maintained until 11 days after ovulation, when the values fell sharply to follicular phase levels. This pattern can be seen in Figure 3.4.

Our control group was also compared with that of Bäckström et al (1976b). The curve for our control group was more 'spiky', indicating the small sample, but the basic shape is the same (Fig. 3.2). The chief difference is the higher level found by Bäckströms group at the two low points of oestradiol synthesis, after the mid-cycle peak and at menstruation. As their assay was not specific for oestradiol, the oestrogen levels that they measure will also include oestrone and possibly other oestrogens. The amount of oestrone produced by peripheral conversion of androstenedione is relatively constant throughout the cycle, so that oestrone will make a relatively larger

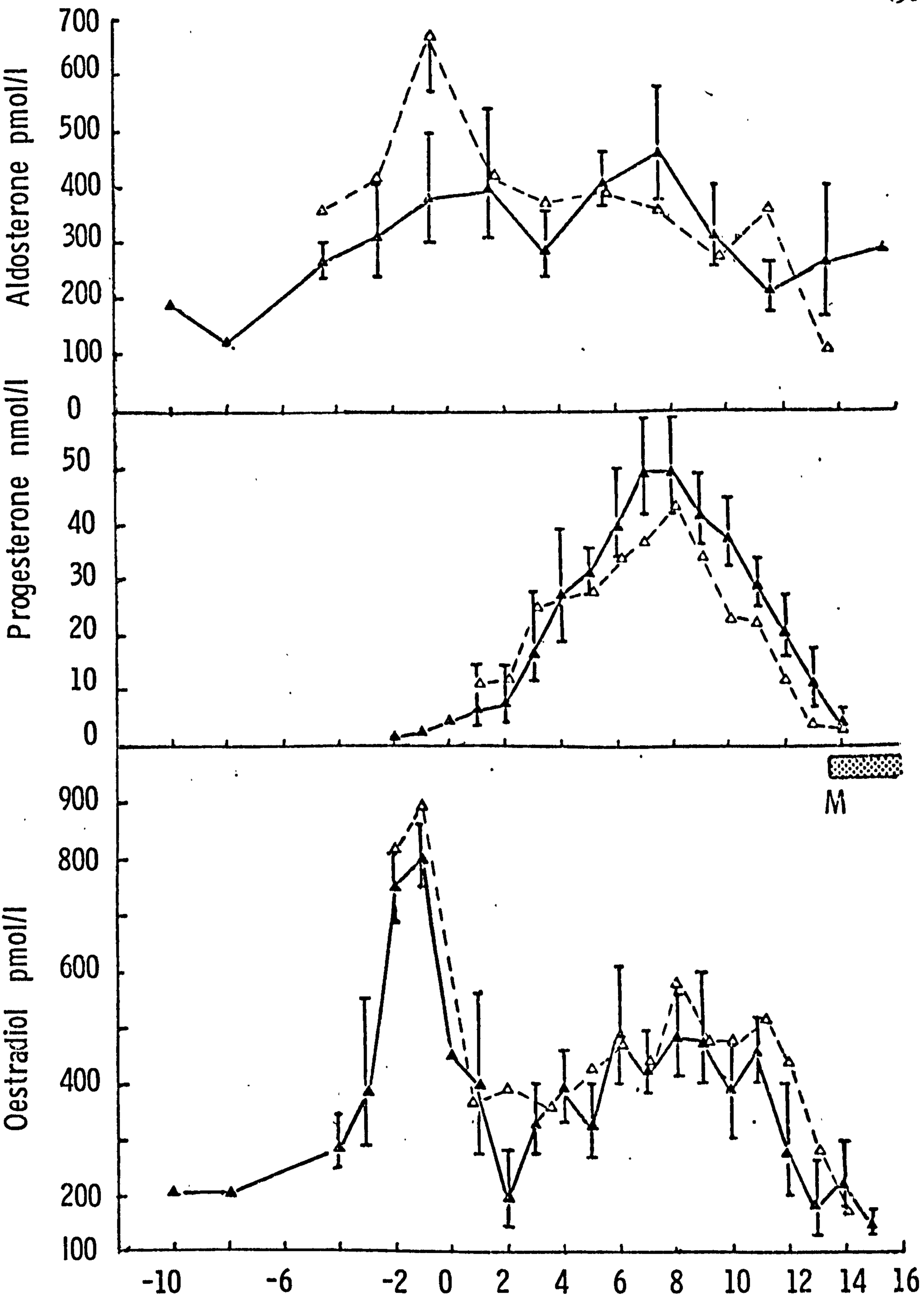


Figure 3.4. Daily log. mean(±S.E.) plasma aldosterone, progesterone and oestradiol levels , arranged around the day of the rise in basal body temperature, in 20 PMS cycles (Δ- - -Δ) and in ten control cycles (▲——▲).M denotes the mean day of onset of menses.

Table 3.5. Comparison of Plasma Oestradiol values obtained by different authors for the menstrual cycle.

		Subjects			Plasma Oestradiol, pmol/l mean ± SD, (range)					
Author	Method	No.	mean Age years	mean Menstrual cycle, days	Follicular	Midcycle* peak	Early* luteal	Late luteal	M⁰	M + 1
This Study	RIA	10	28 (22-34)	29.4	244±93 d2-10	750±75 BBT-2	466±168 BBT+5to+8	456±173 d21-26	125±121	191±21
Guerrero et al (1976)	RIA	17	32	28		1305 LH-1	551 LH+5to+8		218	213
Emment et al (1972)	RIA	14			254±206 d1-10		463±243 d11-20	364±199 d21-31		
Korenman et al (1969)	CPB	15			241±52 d1-10		456±182 d11-20	504±131 d21-31		

*The actual divisions of the menstrual cycle were different for each author; days of the cycle are given in each column, BBT = 1st rise in body basal temperature, LH refers to the day of the LH peak, M is the first day of menses.

Table 3.6 Log Mean Plasma Oestradiol Values (and Standard Deviations) Throughout The Luteal Phase, Dated Backwards From Menstruation (M) in PMS Patients and Controls

Day Cycle (-M)	Controls			PMS patients			P
	No. samples	Mean pmol/l	S.D. pmol/l upper , lower	No. samples	Mean pmol/l	S.D. pmol/l upper , lower	
MO,+1	5	144	42 , 32	5	234	82 , 60	<0.05
1-2	8	170	93 , 60	8	269	158 , 99	<0.05
3-4	6	389	213 , 130	15	513	211 , 150	ns
5-6	7	407	210 , 138	12	513	220 , 158	ns
7-8	6	468	94 , 79	10	457	219 , 150	ns
9-10	7	365	136 , 120	9	398	190 , 129	ns
11-12	6	380	130 , 98	5	282	150 , 100	ns
1-4	14	240	186 , 105	23	417	260 , 160	< 0.01
5-8	13	457	174 , 126	22	490	200 , 150	ns
9-12	13	346	130 , 95	14	355	194 , 126	ns

contribution to total oestrogen levels at times of low ovarian activity (Edman & MacDonald, 1976). This might explain the higher levels found by Bäckström et al (1976b) at these times.

PMS group: The pattern in the PMS group was similar to that of the controls (Fig. 3.4) but there were slightly higher levels especially at the times when oestradiol secretion is falling, that is, after the midcycle peak and at menstruation. The latter difference can be seen more clearly when the logarithmic means are arranged around the day of the next menstrual period (Fig. 3.3). When the data were pooled into two day intervals the mean levels are significantly higher from four days before menstruation until one day after(Table 3.6).

The ratio of progesterone to oestradiol was also calculated for PMS patients and controls and the results are shown in Table 3.7. The ratio was significantly higher ($P<0.05$) for the last eight days of the cycle in the controls.

Table 3.7 Ratio of progesterone to oestradiol in PMS patients and controls, dated from menstruation (M) and Ovulation(Ov)

Day M -	Controls			PMS			P
	n	mean	SD	n	mean	SD	
1,2	6	52	30	8	23	17	<0.05
3,4	7	72	37	14	46	26	ns
5,6	6	97	29	14	78	28	ns
7,8	8	103	41	10	78	27	ns
9,10	6	102	55	9	50	20	<0.05
11,12	5	72	42	9	88	90	ns
1-4	13	61	35	22	38	25	<.05
5-8	14	100	36	24	78	27	<.05
9-12	11	86	49	18	64	59	ns
+Ov							
+1,2	6	81	69	6	31	13	ns
3,4	6	69	42	5	62	44	ns

Aldosterone

Controls: Aldosterone estimations were carried out on the control cycles and the results compared with those from other authors (Table 3.8). The values show good accord with other published methods, although the standard deviation is somewhat higher. The values are particularly comparable to those of Frölich et al (1976), which is perhaps not surprising as the clinical conditions were similar.

The pattern of aldosterone values during the menstrual cycle showed raised periovulatory and luteal values (Fig. 3.4), but no definite pre-ovulatory peak was seen, in contrast to the findings of others (Frölich et al., 1976). The point of ovulation was not determined accurately via the LH peak in our patients, which may partly explain the discrepancy. Figure 3.4 also shows that aldosterone levels in the controls at menstruation are above the values found in the early follicular phase, with a high degree of variability.

PMS Group

The aldosterone values throughout the cycles were not markedly different from the controls (Fig. 3.4) except that values were higher in the pre-ovulatory phase. However the observations at this point were few and the difference was not statistically significant.

As there were few samples per day the data were grouped into two-day intervals around the onset of the next period (Fig. 3.5). At 12 to 14 days premenstrually aldosterone levels are declining, presumably after the peri-ovulatory peak. A second peak is seen at 5-9 days premenstrually in both PMS subjects and controls, with no statistical difference between the values in the two groups. Values

Table 3.8 Comparison of plasma aldosterone levels during the menstrual cycle measured under different conditions

	Method		Subjects			Aldosterone pmol/l (mean ± S.D)		
	Chromatography	Assay	No.	Diet	Conditions	Follicular phase	Midcycle peak	Luteal Phase
This Study	LH-20	RIA	10	ad lib	supine 30 mins.	284±72	(time of ↑BBT) 482±100	400±200 (BBT+2to+12)
Frölich et al (1976)	paper	RIA	7	ad lib	ambulatory	280 LH-10to-3	417	445 (LH+5to+10)
Michelakis et al (1975)	none	RIA	5	defined	supine overnight	183±53		330±80
Schwartz & Abraham (1975)	Celite columns	RIA	3	ad lib	ambulatory	250±41		352±60

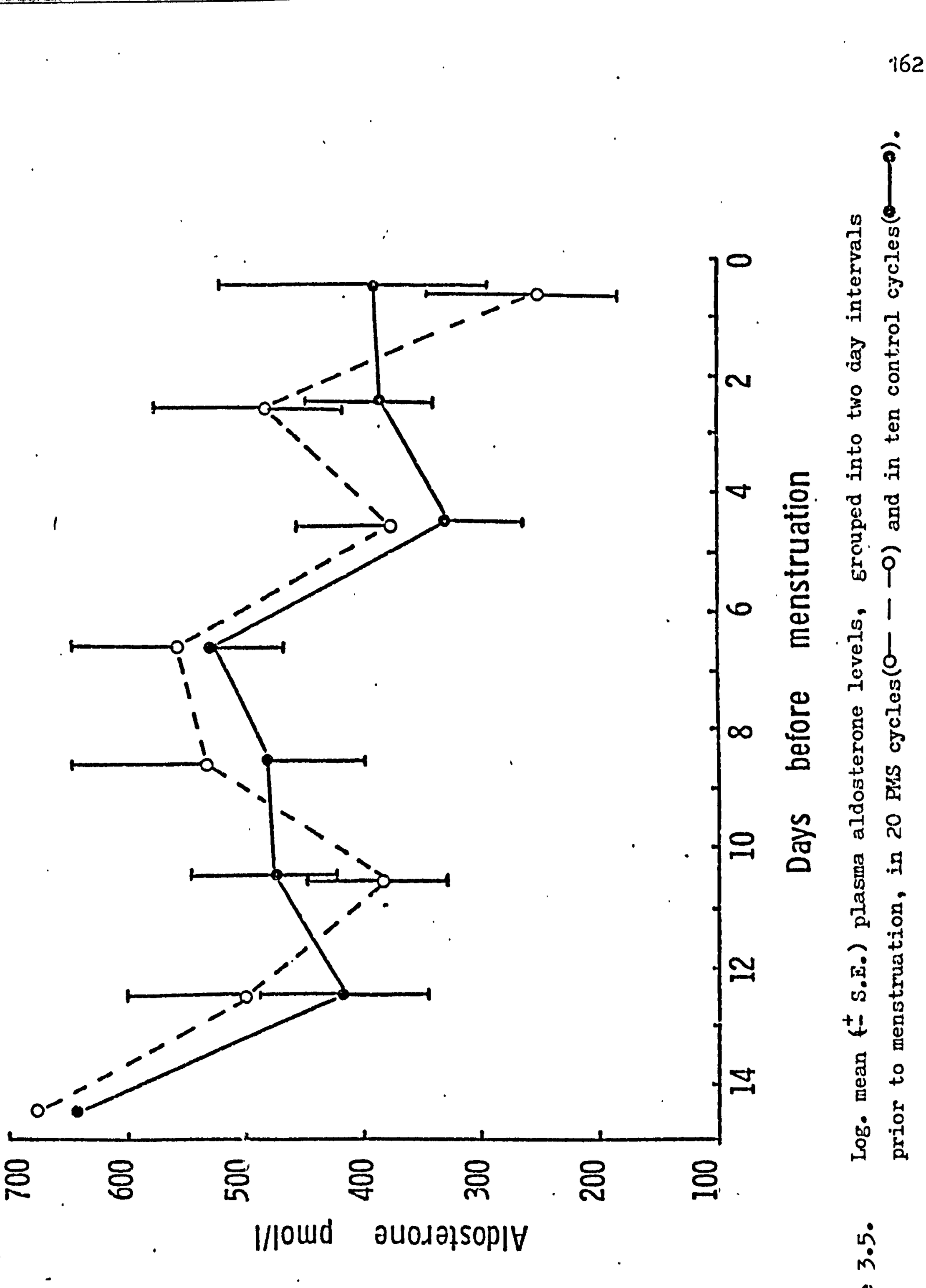


Figure 3.5. Log. mean (± S.E.) plasma aldosterone levels, grouped into two day intervals prior to menstruation, in 20 PMS cycles(O— — —O) and in ten control cycles(●——●).

are still highly variable at the end of the cycle, and although no statistical differences were found, there were unfortunately rather few PMS samples at this stage of the cycle upon which to base any conclusions.

3. DISCUSSION

Ovarian steroids

Firstly it is important to show that systematic errors, such as nyctohemeral rhythms, are not affecting the results. There is now evidence that progesterone has such a rhythm (Florensa et al., 1976), with higher values at 8.00 to 16.00 hours and a decline at 20.00 hours, although this is unlikely to influence our results as the samples were taken between 10.00 and 15.00. hours.

Lenton, Cooke, Sampson & Sexton (1978) have performed a more intensive study of oestradiol variation, under controlled conditions at 15 minute intervals throughout the day. They found that oestradiol levels were markedly raised between 8.00 and 10.00 hours, although this was also the time at which their study started, so that a stress effect cannot be ruled out. In our series one subject, a control, had samples taken before 10.30 h, which should not bias the differences we have observed.

Lenton & coworkers also found a high variability between consecutive samples - up to 57% in the luteal phase - which could not be accountedfor by intraassay variability alone, suggesting that oestradiol is secreted in a pulsatile manner. This might explain the high variability seen in the oestradiol values in the luteal phase in our study, which tends to give a 'spiky' appearance to Figs. 3.3 and 3.4.

Despite these problems the results for progesterone and oestradiol are comparable to those obtained during the menstrual cycle by other authors. Furthermore in a study of women with premenstrual syndrome and controls, Bäckström et al (1976), studying the last ten days of the cycle, found that progesterone was lower in their PMS group from day 10 to day 4 premenstrually, which is in close agreement with our findings. They also found that oestrogen levels were lower in the PMS group eight and nine days premenstrually but that they became significantly higher than the controls from one to five days premenstrually. There was some indication that this 'shift' in oestradiol levels was occurring in our PMS group. The significance levels for the differences are less marked in our work compared to that of the Bäckström's group, and this is probably due to their greater number of daily samples, and also due to the Swedish group measuring oestrogen rather than oestradiol which may mean that they are highlighting differences in other steroids as well.

It is perhaps not surprising, in view of the very slight differences that we have seen, that Loraine & Bell (1971) Prill & Krüger (1963) and Adamopoulos et al. (1972) could find no striking abnormalities in urinary oestrogens and pregnanediol. Some plasma studies have also produced inconclusive or contrary findings. For instance, O'Brien and Symonds (1977), in a very brief report, found that the luteal progesterone peak in a symptomatic group was greater than in an asymptomatic group, and Smith (1975), in a preliminary study, found that progesterone was lower in the last nine days of the cycle in women with premenstrual depression, but oestradiol levels were unchanged. There were however no details about methods, so it is difficult to evaluate these two reports. Smith stresses that there

was considerable overlap between the two groups, and casts some doubt as to whether the hormonal changes are aetiologically important. The results that we have found for Group I do to some extent confirm this view. Although the mean values of progesterone and oestradiol were significantly different, overlap occurred at all stages of the cycle between the PMS and control series.

There are several possible reasons for these equivocal results. The first, and most obvious explanation would be method variability. An interassay coefficient of variability of 19% in the case of progesterone and 10% for oestradiol means that differences between the groups must be greater than 38% and 20% respectively to become at all meaningful. An added difficulty is that there are large episodic fluctuations of plasma levels within the individual during the luteal phase, which makes it difficult to pool data from different days of the cycle, although this would be desirable sometimes when the numbers for each day are too small for statistical analysis.

Several physiological explanations may possibly account for the widely variable values found within the PMS group. For instance, there may be, within the sample of PMS sufferers, two or more separate groups, which may be hormonally dependent in different ways. One hormone-dependent group will tend to have the now classical decrease in the progesterone/oestradiol ratio premenstrually, which was seen in our group in the last eight days of the cycle and which occurs to a variable extent in other published studies (Bäckström & Carstensen, 1974; Bäckström et al., 1976; Smith, 1975). If there are two or more aetiologies within PMS the dividing factor may be the symptoms. In fact, apart from one woman who complained chiefly of headaches and one complaining of gross oedema, all the

patients in Group I complained chiefly of irritability and depression, but Group II were rather more heterogeneous and the question of symptomatology is therefore discussed with regard to the latter group in the next Chapter.

It is also possible that the same changes are occurring in all the women, and that subtle changes in the ovarian steroids detectable in some women in peripheral blood but not in others, may lead to a 'cascade' effect on other hormones, which will in turn lead to the symptoms. Aldosterone could be such a 'secondary' hormone.

Aldosterone

Aldosterone levels in plasma are subject to acute changes brought about by changes in diet and posture, so that standardisation of these two factors is very important. The question of posture is critical as different control mechanisms of aldosterone secretion seem to take place in the upright and supine positions. In subjects who are supine overnight the nyctohemeral rhythm of aldosterone follows that of cortisol and the correspondence with renin activity is weak (Katz, Romfh & Smith, 1975) although suppression of ACTH and cortisol does not suppress the aldosterone pattern. The increase in aldosterone upon upright posture is thought to be due to increased renin activity, controlled by the redistribution of the circulating blood volume (Sassard, Vincent, Annat & Bizollon, 1976).

Thus while supine, it appears that aldosterone is under the control of a central ACTH-like control, with perhaps a role for potassium; while upon orthostatism, renin becomes the important controlling factor. This disparity is underlined by the different patterns obtained in the menstrual cycle, in that a mid-cycle peak of aldosterone occurs under upright but not supine conditions, (see

Chapter 1).

Unfortunately our results are not strictly comparable with either the 'upright' or 'supine' groups of other studies. However Sassard et al (1976) showed that aldosterone is markedly increased in the upright position and that the levels do not decrease after 30 minutes' recumbency, which would tend to class our studies in the 'upright' group. These workers also found that renin activity in plasma was significantly decreased after 30 minutes' recumbency, and they explain these findings in terms of the lower metabolic clearance rate of aldosterone and also the possibility of a persistent stimulus such as potassium.

It appears quite valid to compare our results with those of workers studying 'upright' subjects, and indeed the values agree quite closely (see Table 3.8).

However we did not see a well defined pre-ovulatory peak, as described by Frölich et al. (1976) although aldosterone levels in both PMS and control subjects rose one or two days before ovulation and fell again after ovulation. In this respect the pattern is closer to that of Sundsfjord & Aakvaag (1973) who found a gradual pre-ovulatory increase in aldosterone levels.

Plasma aldosterone levels in the luteal phase of both groups were raised for five to ten days after ovulation, and this increase, which is about double the levels in the early follicular phase, is close to the findings of others (Table 3.8). In the control group aldosterone levels were still higher than follicular levels at menstruation (Figs 3.4 & 3.5). There is no mention of this by other authors, perhaps because most attention has focussed on the ovulatory phase of the cycle. Reich (1962) did however note an increase in aldosterone excretion on certain days of menstruation in

some of her subjects, and she mentioned that this could be an ACTH-evoked response to stress.

It is perhaps not surprising, in view of the complexity of the control of plasma aldosterone, that no statistically significant differences were found between the PMS and control subjects under these relatively uncontrolled conditions. In a study of one patient with premenstrual oedema (Schwartz & Abraham, 1975) the patient had very high luteal phase aldosterone levels, although follicular phase levels were quite low, and this fits well with the hypothesis of Janowsky et al., (1973), that aldosterone is responsible for water retention in PMS. Yet the one patient in our series with premenstrual weight gain of six kg with oedema did not have a marked luteal increase in aldosterone. Oelkers et al (1973) studied three women with cyclical oedema of more than three kg and found decreased aldosterone with renin excretion, despite sodium retention. They postulated that another salt-retaining factor is responsible, although it is perhaps also likely that the excretion rates are not reflecting serum levels due to decreased clearance or increased conversion to other metabolites. However, cyclical oedema may be a special case in that most patients with 'classical' PMS only gain 1.4 kg or less premenstrually (Bruce & Russell, 1962).

Indeed, in our series with 'classical' PMS we could find no abnormalities in aldosterone levels, in agreement with a preliminary report by O'Brien & Symonds (1977). These workers did however find that spironolactone, a steroid analogue of aldosterone and hence an inhibitor of aldosterone action, alleviated symptoms in a double-blind trial. Spironolactone does have other endocrine effects, such as the elevation of progesterone levels, possibly via the adrenals

(Stripp, Taylor, Bartter, Gillette, Loriaux, Easley & Menard, 1975).

It is interesting that the only marked increase in aldosterone levels in the PMS group occurred prior to ovulation, although this difference was not significant. The origin of the pre-ovulatory increase is uncertain. Both plasma renin activity and substrate are unchanged at this time, (Sundsfjord & Aakvaag, 1973) suggesting that if oestrogen is responsible, it is acting outside the renin-angiotensin axis, possibly by direct action on the adrenals. It has been suggested that 17α-OH progesterone, which also has a midcycle peak (Strott et al., 1969), may be involved although a natriuretic effect has not been demonstrated for this hormone (Landau, Lugibihl & Dimick, 1958).

A recent study of fasting supine aldosterone, renin activity, renin substrate, oestradiol, progesterone, gonadotrophins and prolactin in seven women throughout the menstrual cycle (Kaulhausen, Leyendecker, Benker & Breuer, 1978) has identified a pre-ovulatory peak in aldosterone and renin activity even in a few supine individuals, but changes were on the whole inconsistent, and no clear relationships emerged, which is disappointing.

It is possible that ACTH, which increases around the day of ovulation (Genazzani, Lemarchand-Beraud, Aubert & Felber, 1975) may be involved in a pre-ovulatory peak.

The results for the luteal phase do not point to any conclusive role of aldosterone in the aetiology of PMS. It is possible though that absolute levels of aldosterone in plasma are less important than the ratio between aldosterone and progesterone, which acts as an aldosterone antagonist in the luteal phase (see Chapter 1). The

progesterone/aldosterone ratios for PMS subjects and controls are shown in Table 3.9

Table 3.9

Ratio of Progesterone to aldosterone in PMS and controls dated from menstruation (M) and ovulation (ov).

M-days of cycle	Controls (8)			PMS (8)			P
	No.	Mean ratio	SD	No.	Mean ratio	SD	
1-4	12	42	25	11	47	32	ns
5-8	13	116	71	11	63	19	<.05
9-12	11	67	38	9	51	36	ns
ov+							
1,2	6	41	62	4	28	13	ns
3,4	5	59	44	4	62	56	ns

It might be expected that if the ratio is an important factor, the degree of statistical significance in the differences between groups will be greater for the ratio than for either hormone alone. However this is not the case.

There is another element to the study of aldosterone plasma levels which has recently come to light. It has been shown that oral contraceptives increase the binding of aldosterone to a specific binding protein, aldosterone binding globulin (ABG), and that the degree of binding is related to the oestrogen content of the combination (Nowaczynski, Murakami, Richardson & Genest, 1978). As our method measures total steroid, any changes in binding would not be seen, although it may be only the free steroid that is physiologically active.

Even if aldosterone levels in peripheral plasma are not important, there could still be effects of the luteal rise in aldosterone occurring at the tissue level. The retention of sodium or water or both may directly affect brain function. There is some evidence that the distribution of sodium and water are altered in chronically depressed patients (Coppen & Shaw, 1963) although the distribution returns to normal when the patients recover. The other possibility is that aldosterone metabolism directly affects brain function, and although there is no evidence for aldosterone itself being involved, angiotensin does appear to alter animal behaviour and autonomic function. For instance it affects the uptake and release of norepinephrine by the brain in rats and it counteracts the anaesthetic effects of amobarbitol (Palaić & Khairallah, 1967).

Thus the findings for Group I although not ruling out the involvement of the renin-angiotensin-aldosterone system, would tend to make a major role seem unlikely. A role for ovarian steroids seems probable in view of the altered progesterone/oestradiol ratio seen for eight days premenstrually, and this is discussed further in later chapters.

CHAPTER 4

Plasma progesterone in relation to prolactin, symptoms and psychological profiles in PMS.

1. GROUP II PATIENTS

Protocol

A large group (105) of women aged 19 - 47 years came to the clinic after reading a magazine article about the research into PMS in our Department. At the first clinic visit each woman was asked to record details of her menstrual cycle and any medications taken, to rate which were her 'most troublesome' symptoms, and to record any other symptoms noticed. From this self-rating the symptoms from each woman were grouped into the clusters seen by Moos (1968) in his survey, and modified by Clare (1977)(see Table 4.1.). These ratings were checked as far as possible with the womans daily symptom records.

In addition 58 of these women, chosen at random, were asked to complete the General Health Questionnaire, which identifies non-psychotic psychiatric ill-health and is a general indication of psychiatric distress (Clare, 1977). The completed questionnaires were analysed by Dr A.W. Clare of the Institute of Psychiatry.

At least three blood samples were taken during the luteal phase, on about days 18, 21 and 25 of a 28 day cycle, with a shorter or longer interval for cycles of less than or more than 28 days respectively. The blood samples were taken under conditions described in Chapter 2, section 4, and analysed for progesterone. During the course of this study we became interested in the role of prolactin in PMS, and as a consequence 77 women had serum prolactin levels measured. A computer estimate of the progesterone index was also made in these 77 women, using an arbitrary luteal phase of 14 days.

Characteristics of the patients

The mean age of the women was 34.1 ± 6.2 years and the mean length of menstrual cycle was 28.0 ± 3.7 days (range 24 to 33 days). The symptoms reported by the women were grouped into clusters of symptoms and the incidence of each cluster is shown in Figure 4.1. Most women experienced symptoms from more than one cluster, and a few women had symptoms in up to four clusters.

Table 4.1 Symptom clusters (see Clare, 1977) experienced by 105 women complaining of premenstrual syndrome

Symptom cluster	Example of complaint	No. women with cluster (%)
Pain	Headache, tiredness	48
Concentration	Confusion, Clumsiness	20
Behavioural change	Loss of efficiency	1
Autonomic reaction	Dizziness, Cold sweats	12
Water retention	Painful breasts, bloating	70
Negative affect	Irritability, Depression	95
Arousal	Feeling of well-being	0
Control	Chest pains, tingling	1

The onset of PMS was reported by the women to have occurred at puberty in 28%, after childbirth in 25%, and spontaneously in the previous two to ten years in 30%. Six women (6%) mentioned the contraceptive pill as a precipitating factor, and 3 other mentioned miscellaneous factors such as sterilisation, abortion and marriage. The time of onset was not recorded in 7%.

The mean duration of symptoms was 10 ± 4 days with a range

of 2 to 14 days, as recorded on the retrospective questionnaire. In 103 women the symptoms ended by day 3 of the next cycle, so that in only two were the symptoms prolonged beyond this. 59% of the women were parous.

2. HORMONE LEVELS IN GROUP II

Plasma progesterone levels

The progesterone values for 92 subjects (excluding the 13 already studied in Group I) were arranged into daily log means around the onset of the next period, and the results are shown in Fig. 4.1. There was no statistical difference between Group II and the controls for Group I, although the values for the PMS group are slightly lower.

The progesterone values for each individual were also plotted and the general curve for each woman compared with the composite curve for the controls, with confidence limits, seen in Fig. 4.1. It was found that 30% of the subjects had a 'low' progesterone curve, which is defined as a curve where two or more points fall below the 90% confidence limits of the control curve. 55 (60%) had a progesterone curve within the normal limits, and 9 (10%) had values above the confidence limits of the controls.

In the 77 women with a computer index of the progesterone peak the mean value was 38.9 $\pm$ 9.1 (SD) nmol/l, while that of the Group I controls was 43.1 $\pm$ 7.4 (SD) nmol/l (not significant).

Serum Prolactin Levels

Prolactin estimations were carried out in the luteal phase in 77 patients from this group, of whom eight had more than one sample assayed from the luteal phase and 20 had an additional sample measured in the follicular phase. The mean prolactin level per

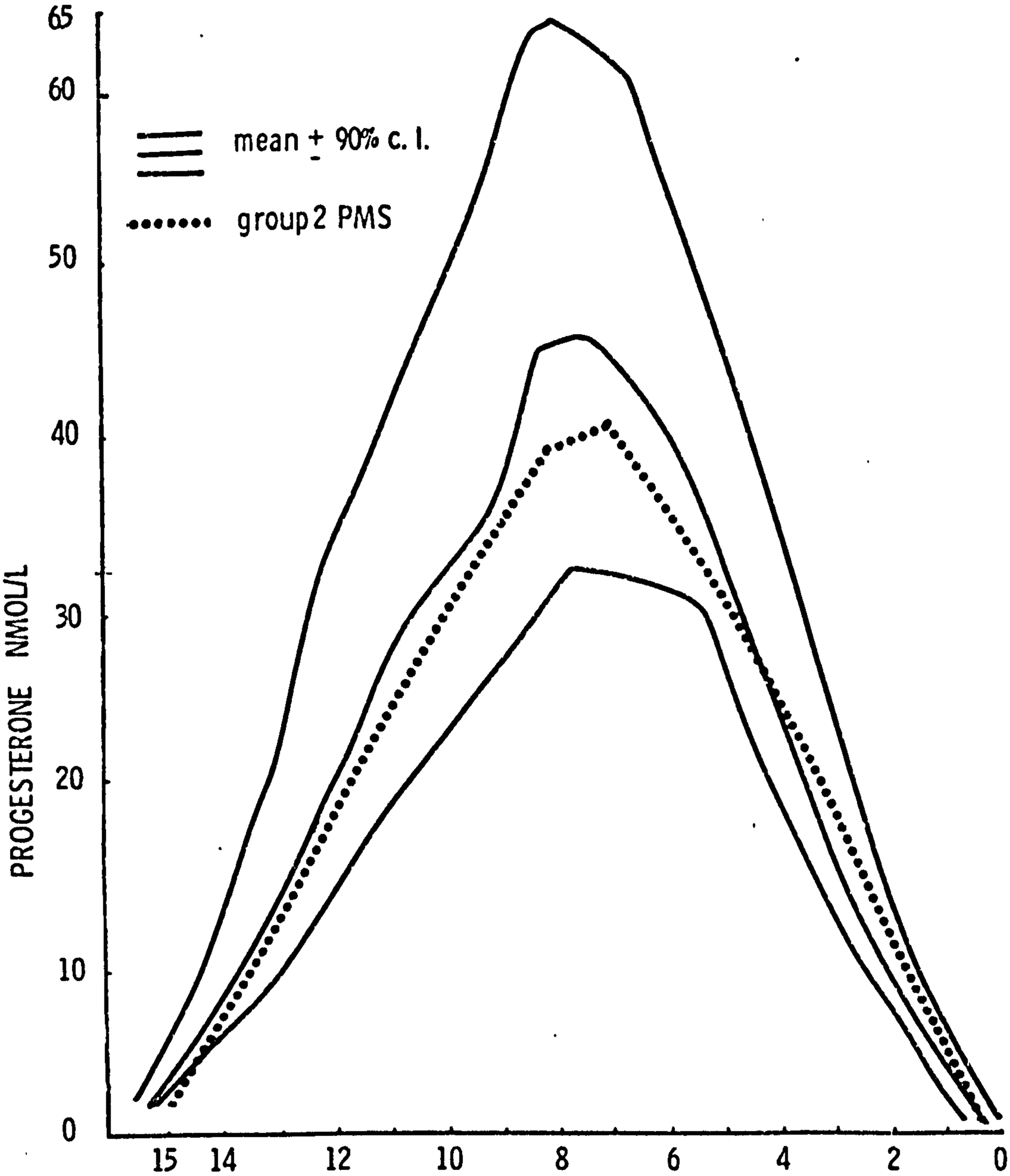


Figure 4.1. Log. mean plasma progesterone levels in Group II PMS patients (●●●●●●●●) compared with the log. mean curve ± 90% confidence limits for 10 control cycles(——).

patient in the luteal phase was 244 ± 100 (S.D) miU/l, (range 80-500 miU/l) excluding two patients with values of 800 and 900 miU/l. The range for normal women in this assay is 0-480 miU/l, so that the majority of PMS patients have apparently 'normal' prolactin levels.

Prolactin estimations were made throughout the luteal phase, so that a spread of values was obtained for the last two weeks of the cycle, although there were few values to represent each individual day. The pattern of values for the cycle (Fig. 4.2) shows apparently no marked increase in the second half of the cycle, at least not until the last day before menses. The data represented in this way did not show a marked mid-cycle peak, but there were rather few samples around this time of the cycle.

The relationship between progesterone and prolactin levels

Prolactin estimations were carried out in 42 women with 'normal' progesterone and in 33 women with 'low' progesterone, as defined above. In Figure 4.3 there is a comparison of levels of luteal phase prolactin in patients with 'low' and 'normal' progesterone. The mean prolactin value was 279 ± 104 miU/l (SD) (n = 33) for the low progesterone group and 215 ± 104 miU/l (n = 42) for the normal progesterone group. Thus the low progesterone group tended to have higher prolactin levels ($p < 0.01$) in the luteal phase. The two patients in Group II with hyperprolactinaemia (>500 miU/l) both had regular cycles , although one patient had a low progesterone peak, as defined above, and the other had a peak value of 38 nmol/l progesterone but a short luteal phase of ten days.

The distribution of prolactin throughout the cycle also appeared to differ in relation to progesterone status (Fig. 4.4), although there were not enough values to separate the follicular phase values in this way. The difference in the prolactin seen in

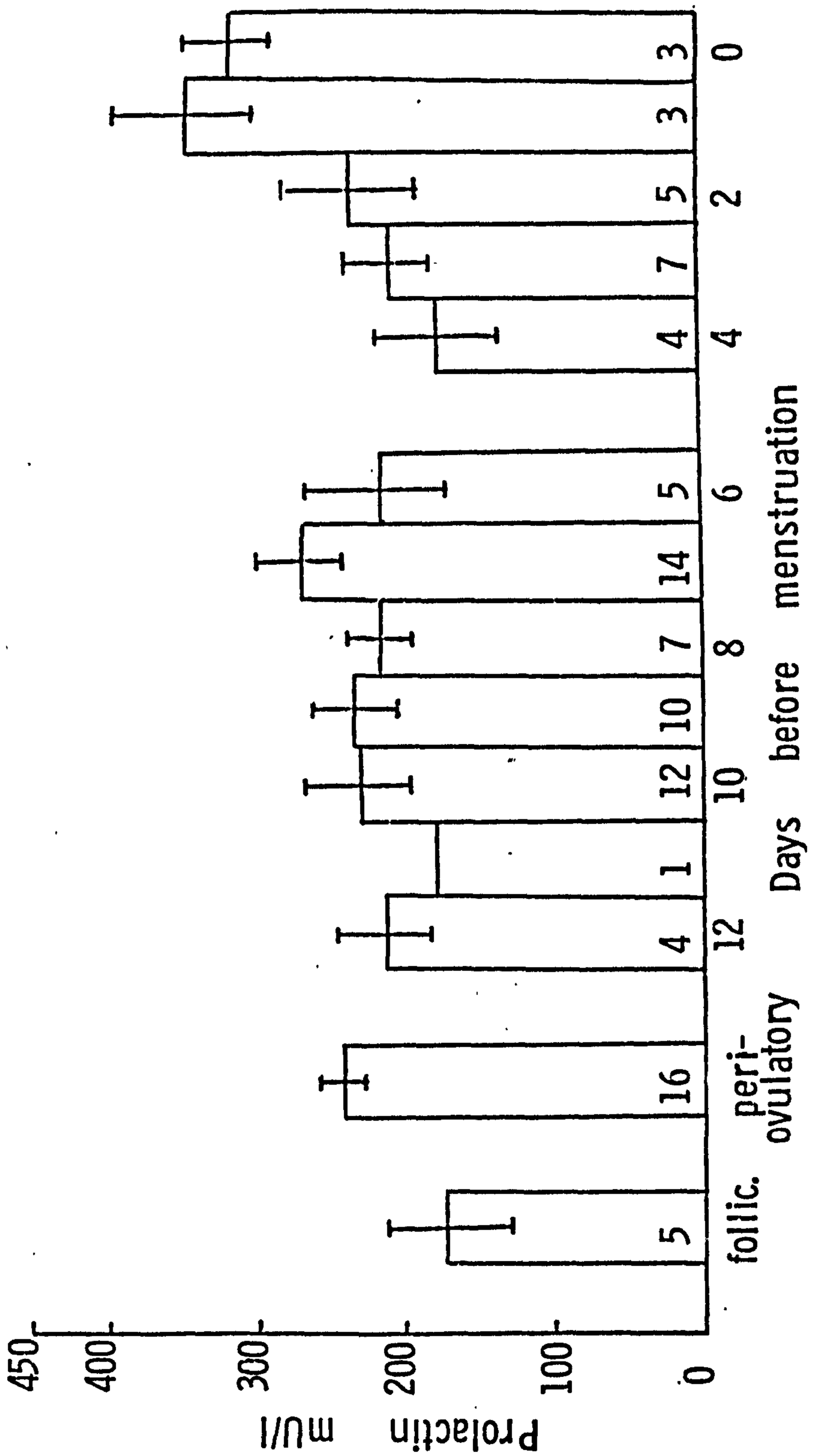


Figure 4.2. Mean (± S.E.) serum prolactin levels during the menstrual cycle in women with PMS. The figures at the base of the columns are the numbers of samples per day.

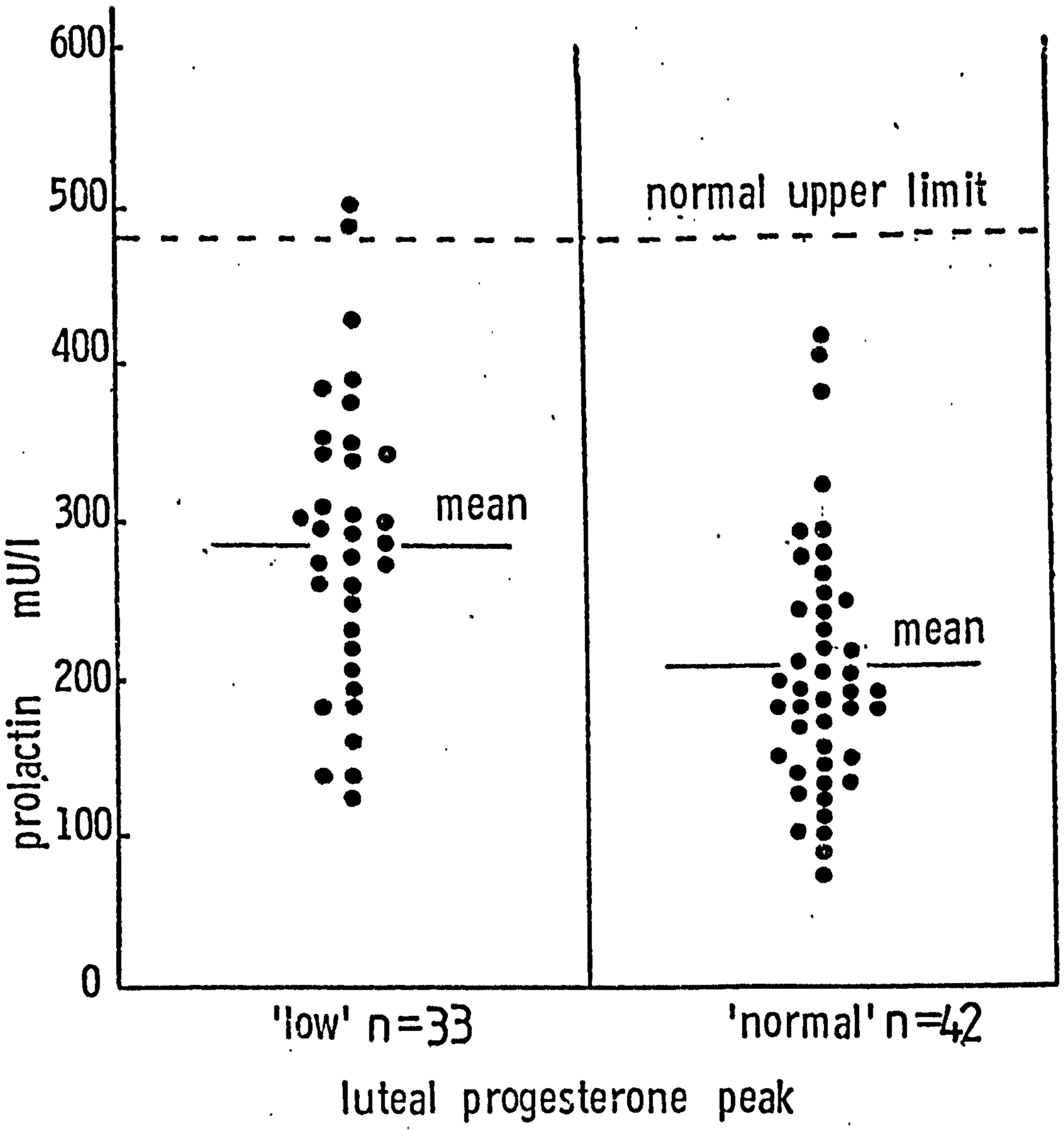


Figure 4.3. Comparison of serum prolactin levels in PMS subjects with a 'low' progesterone curve (see text) and those with a 'normal' progesterone curve.

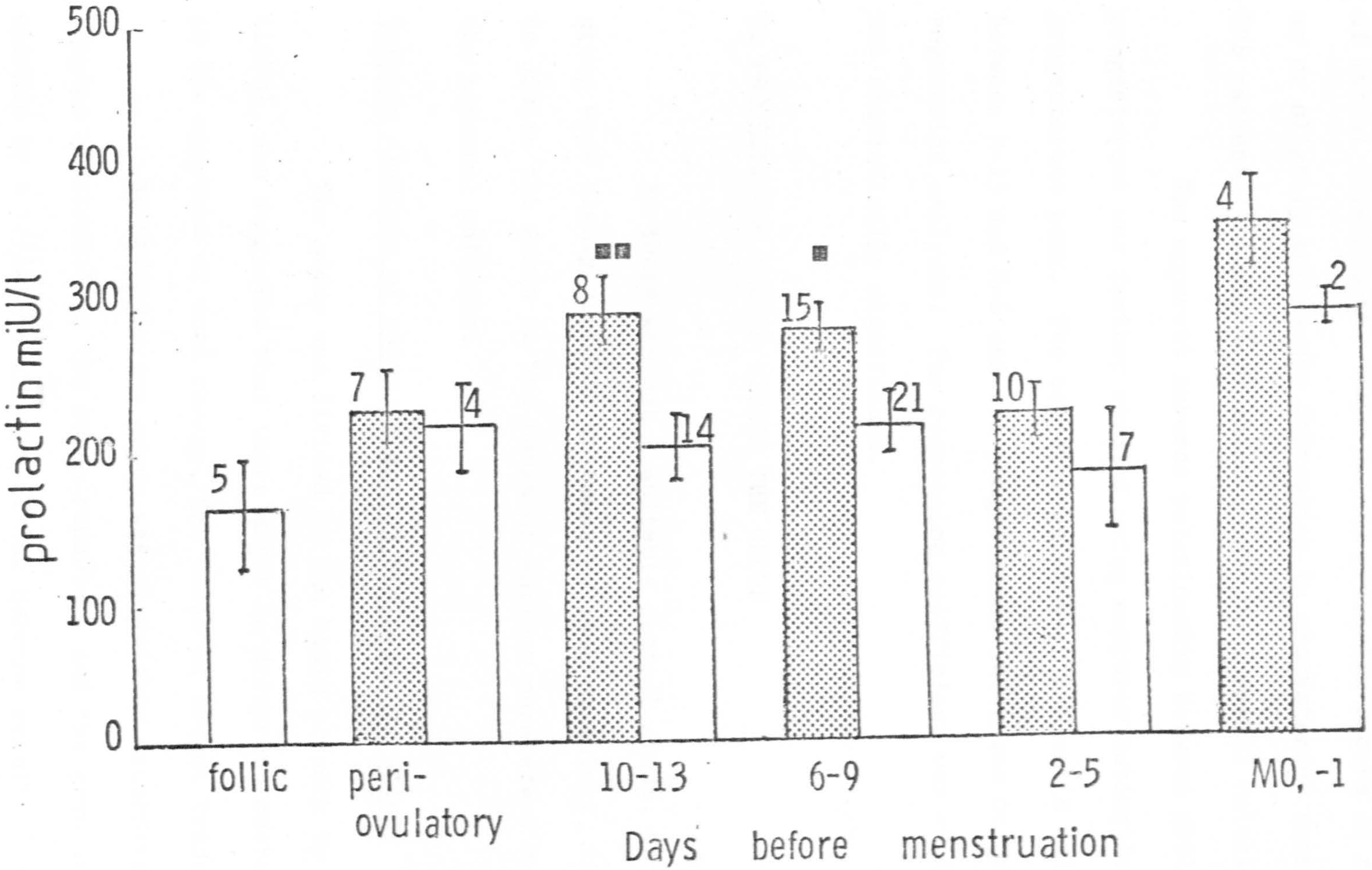


Figure 4.4. Mean (± S.E.) serum prolactin levels during the menstrual cycle in PMS subjects with a 'low' progesterone curve (▨) and those with a 'normal' progesterone curve(□). The numbers at the top of each column denote the number of estimations per day. ■ marks a statistical difference ($P<0.05$) and ■■ indicates a statistical difference where $P<0.01$

Figure 4.4 was not significant in the periovulatory or late luteal phase (from M-5 to MO), although this may be partly due to the small numbers of samples at this stage of the cycle. However prolactin levels were markedly raised from M-13 to M-6, which is the time of luteal development. Thus raised prolactin occurs at the same time as or slightly before the depression in progesterone seen in some PMS patients (Figure 3.3) which points to a causal relationship.

The apparent inverse relationship between prolactin and progesterone was further studied using computer estimates of the progesterone peak. The correlation between prolactin values at between M-13 and M-6 and the progesterone index was calculated by regression analysis. The regression coefficient was -0.27 which is not statistically significant.

3. PROGESTERONE VALUES WITHIN THE GROUP

As there was not a suitable control group, and as the PMS group were highly variable in terms of symptomatology, it was decided to divide the women on the basis of various parameters and to examine the hormonal patterns.

Symptom clusters of water retention and negative affect

The group was divided on the basis of work by Cullberg (1972), who suggested that there might be different mechanisms involved in the symptoms of mood change, and those of breast tenderness and oedema.

Accordingly our group was classified according to the symptom clusters that the women reported, and the groupings were checked by a colleague unaware of the hormone results.

1. 70 women (67%) complained chiefly of 'negative effect' premenstrually. This symptom cluster includes crying spells, anxiety, restlessness, irritability, mood swings, depression and tension.

However 51 of these women also reported water retention symptoms.

2. 27 women (26%) complained chiefly of the water retention cluster, usually breast tenderness, gain in weight, or feelings of bloatedness (and also skin disorders). Again, 24 of these women also experienced negative affect.

3. There were eight women who fitted into neither category, either because their records were unclear or because they recorded a multiplicity of symptoms.

There was no difference in mean age or the length of the menstrual cycle in the two groups. The log mean progesterone values for each day, dated from the onset of the next period as before, was plotted for both groups, and the results can be seen in Fig. 4.5. Progesterone levels were lower in the 'negative affect' group from M-3 to M-10 in the figure but this difference was statistically significant only from days M-7 to M-8 ($P < 0.01$).

When the mean value from the control group are compared (see Fig. 4.1) the 'negative affect' curve falls below the control curve, while the 'water retention' curve is above it.

No other difference could be seen for the 'water retention' and 'negative affect' groups apart from a slightly different pattern in the onset of the symptoms (Table 4.2).

Table 4.2 Characteristics of women complaining chiefly of 'negative affect' (N.A.) and 'water retention' (W.R.)

	N.A. group(70)	W.R. group(27)
Mean age (years)	34 ± 6	36 ± 7
Duration symptoms (days)	10 ± 4	10 ± 3
Onset: childbirth	20 (29%)	3 (11%)
(no.) Puberty	21 (30%)	7 (26%)
Other	25 (36%)	16 (59%)
Unknown	4 (6%)	1 (4%)
No. parous	45 (62%)	16 (55%)
Mean parity	1.2	1.3
Prolactin miU/l	243±96	232 ± 86

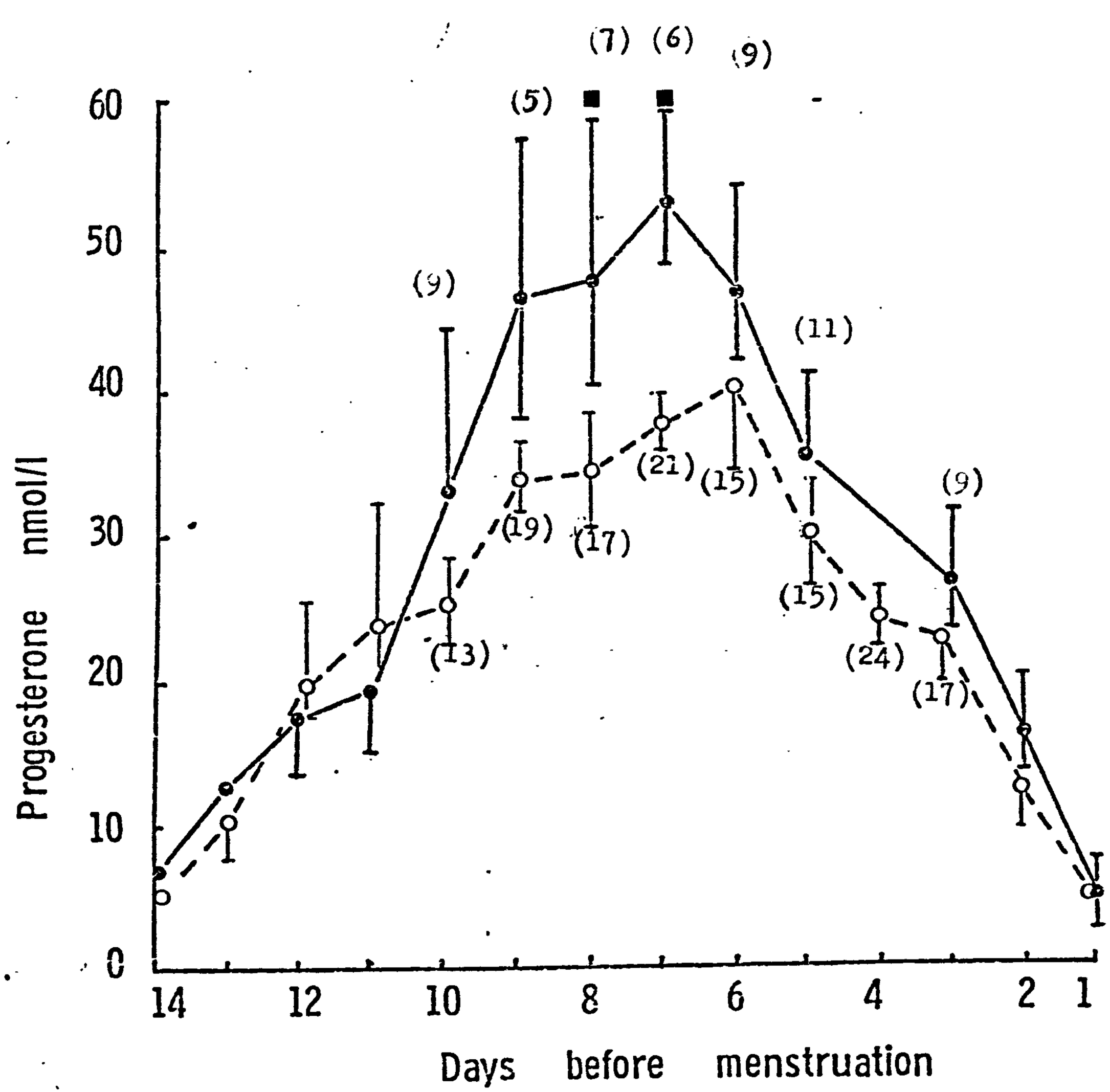


Figure 4.5. Log mean(± S.E.) plasma progesterone values prior to menstruation in PMS patients with main complaints of 'water retention' (●——●) and 'negative affect'(o— — — —o). Numbers in parentheses are the number of samples per day. ■ indicates statistical significance ($p<0.05$).

Unfortunately it was not possible to examine the relationship of progesterone to the absence or presence of any particular symptom, such as breast tenderness or headaches, as specific questions were not asked about these.

Psychological profiles using the General Health Questionnaire

Not surprisingly all the women (58) recorded a positive PMS value on a menstrual distressquestionnaire provided by Dr. Clare (Clare, 1977). 26 women (45% of those who completed the questionnaire) had a positive score on the General Health Questionnaire, indicating some neurotic illness, while the incidence found for all women of this age group attending a general practice is about a third (Clare, 1977). The mean progesterone values for the 'neurotic ' and 'non-neurotic' groups were calculated as above but no differences could be seen (Fig. 4.6).

The stage of the cycle at which the GHQ was completed did not seem to influence the score on the questionnaire. 27% of the GHQ positive group completed the GHQ during days 20 to day 3 of the cycle, compared to 28% of the GHQ negative group. Thus being 'premenstrual' at the time of answering did not seem to influence the response.

Other aspects of these patients were compared, such as age, menstrual cycle length, and onset of symptoms, but no significant differences were seen. The pattern of symptoms experienced by the women was also similar in both groups.

Prolactin estimationswere available in 33 of the women who had undergone this psychiatric assessment. The mean prolactin value for the 'non-neurotic' women was 272 $\pm$ 104 miU/l (n = 17), and the mean level for the 'neurotic' group was 265 $\pm$ 339 miU/l (n = 16). However this latter group included one patient with hyperprolactinaemia

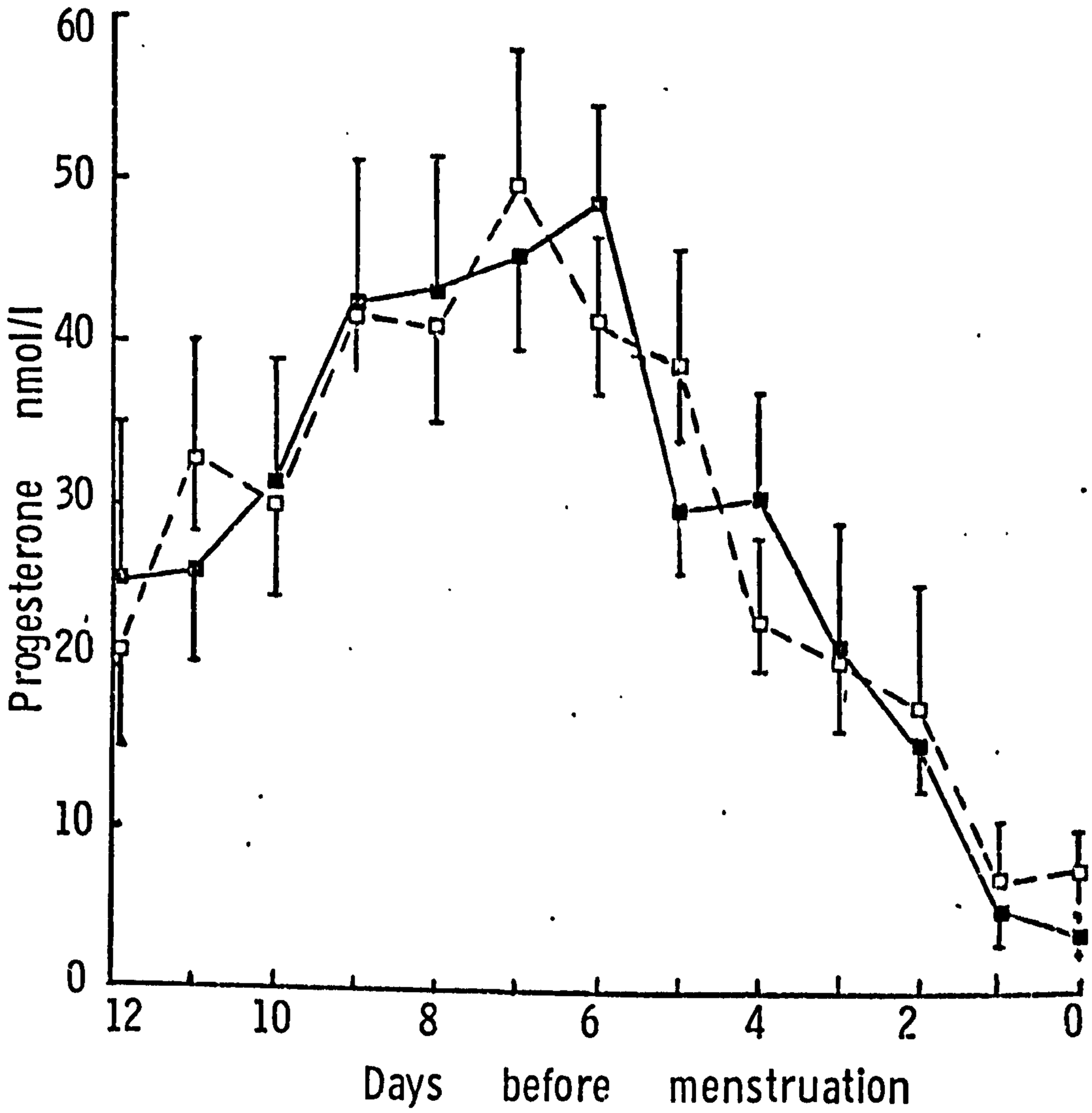


Figure 4.6. Log mean (± S.E.) plasma progesterone prior to menstruation in PMS subjects with a negative (■——■) and positive (□- - -□) GHQ.
n is a mean of 6 ± 2 for all points in both groups.

of 900 miU/l, and if this figure is excluded, the mean for the GHQ positive group becomes 183 ± 63 miU/l, which is significantly lower ($p < 0.01$) than the GHQ negative group.

4. DISCUSSION

Our group was slightly younger than a sample of 100 patients described by Dalton (1977); 34% of our group were aged 21-30 compared to 19% of Daltons group. The source of referral may be relevant, since Daltons group were difficult cases referred by G.P.'s.

Although there is no generally agreed symptomatology, it is believed that the symptoms of depression, irritability and tension are almost always present, and this is confirmed in Group II where 95% complained of mood changes. The incidence of 70% for water retention, which includes breast tenderness, agrees with other recorded series (see Table 1.1). The onset of PMS has not been recorded in most studies although some data have been obtained by Greene & Dalton (1953) which seems to agree with our results.

In summary, although Group II came to us in an unusual way, via a magazine article, there is no reason to believe that they were atypical of PMS sufferers in general. The social class distribution was probably skewed towards classes I and II, reflecting the readership of the magazine, but there is no published study of the effect of social class on PMS.

Progesterone and prolactin

Although the progesterone levels of Group II were slightly lower than those in the controls this difference did not approach

statistical significance, as it did in Group I. One reason for this may be that the samples in Group II were assayed in much later batches so that inter-assay variability may bias the results. However it is interesting that 30% of Group II had a progesterone peak value lower than the 90% confidence limits of the controls, while only 10% would be expected statistically ($P < 0.01$). The expected proportion (10%) of patients had progesterone peaks above the 90% confidence limits of the controls. The lower 90% confidence limit of the peak (at day -7 premenstrually) was 32.6 nmol/l which shows good agreement with the 10 ng/ml (32 nmol/l) level regarded by Johansson (1969) as the criterion of a normal progesterone peak.

It is very difficult to make any conclusions about general levels of prolactin in PMS from Group II as a suitable control group was not available. It may even by misleading to draw conclusions from the pattern of prolactin levels throughout the cycle, as each day is represented by samples from different women. However it can be stated that the vast majority of PMS patients have prolactin levels within the range for 'normal' women - that is, women without any obvious pituitary or ovarian disease. From Fig. 4.2 it appears that prolactin levels are higher in the luteal phase than in the follicular phase and that there is a sharp increase 2-3 days premenstrually. This latter change is more uncertain as very small numbers were involved at this stage of the cycle and a few patients with high prolactin levels throughout could bias the results if they had samples taken only at this time.

McNeilly & Chard (1974) found highly variable patterns of daily prolactin in their group of eight women, with some showing an ovulatory and luteal increase, and others showing no change at all.

It is tempting to speculate that the differences may be due in part to the presence or absence of PMS in the women, and to attach some aetiological significance to the luteal increase seen in our group. Halbreich et al. (1976) in a similar study to our own, did look at a PMS and a control group, and they concluded that the luteal rise of prolactin was more marked in the PMS group (28% compared to 17%). Their design was slightly better than our own in that each patient contributed one follicular and two luteal phase samples, although this is still nowhere near the ideal of daily samples (or more) which would give a far more accurate picture.

In our group there appears to be a mean increase of 34% in the mid-luteal (M-9 to -6) compared to the follicular phase (Figure 4.2). Without a control group this does not confirm the findings of Halbreich and co-workers although it might add a little weight to his findings.

As already described, gross hyperprolactinaemia leads to amenorrhoea and there have also been several recent papers linking prolactin with impaired luteal function, although in few patients. Hyperprolactinaemia has been linked with the short luteal phase (Corenblum et al., 1976) and progressively shorter luteal phases, with associated low progesterone secretion, have been found to precede amenorrhoea when hyperprolactinaemia recurs after bromocryptine therapy (Seppälä et al., 1976). There is some evidence that even slightly raised prolactin levels are associated with luteal phases of 12 days or less in infertile women although there was no corresponding reduction in progesterone secretion (Fredriccson, Björk, & Carlström, 1977).

We were able to show that there is a tendency for slightly raised prolactin levels to precede or coincide with low progesterone secretion, although there was no apparent shortening of the luteal phase. Benedek-Jaszmann & Hearn-Sturtevant (1976) found a similar association between high prolactin and low progesterone (from endometrial biopsy) in a PMS group. These results suggest an inhibitory role of prolactin on progesterone secretion, but it is not possible to conclude whether prolactin is acting directly on the ovary or whether it is inhibiting the luteotrophic effect of the gonadotrophins. The only evidence so far for the direct action of prolactin on ovarian function comes from the in vitro work of McNatty et al. (1974), already described in Chapter 1. It has also been suggested that such a mechanism occurs in vivo (Glass, Shaw, Butt, Logan Edwards & London, 1975; Editorial, 1977).

However in a recent paper McGarrigle, Sarris, Little, Lawrence, Radwanska & Swyer (1978) treated 17 hyperprolactinaemic, amenorrhoeic women with clomiphene citrate and HCG, and obtained progesterone and oestradiol levels indicative of a normal corpus luteum, followed by pregnancy in most cases. Assuming that prolactin levels remained high during and after treatment (although this is not stated by the authors) it appears that corpus luteum formation is not inhibited by hyperprolactinaemia provided that gonadotrophic stimulation is sufficient.

It is already known that high prolactin abolishes the LH surge in response to oestradiol administration (Glass et al., 1975; Aono, Miyake, Shioji, Kinugasa, Onishi & Kurachi, 1976). Yet the pituitary response to LHRH is unimpaired in women with hyperprolactinaemia (Zarate, Jacobs, Canales, Schally, de la Cruz, Soria &

Daughaday, 1973; Mortimer, Besser, McNeilly, Marshall, Harsoulis, Tunbridge, Gomez-Pan & Hall, 1973). This implies that the positive feedback of oestradiol to the hypothalamus is blocked in hyperprolactinaemia, and the work of McGarrigle and co-workers confirms that this is the chief mechanism, since their success with Clomiphene in promoting follicular development as part of their therapy suggests that the negative feedback mechanism is intact. Others have found that Clomid alone is ineffective in hyperprolactinaemia (Bohnet, Dahlen, Wuttke & Schneider, 1975).

According to these findings, the prolactin-associated low progesterone secretion in our group would be due to an inadequate LH peak and consequently a poor corpus luteum, and a direct luteolytic action of prolactin would be unlikely.

However the situation of hyperprolactinaemic anovulation is rather different to the situation in our patients, where ovulation was occurring and the prolactin levels were chiefly within the normal range. Prolactin has biphasic effects human granulosa cells in vitro, depending on the dose, and the binding of prolactin to these cells varies during the menstrual cycle (McNatty et al., 1974). Therefore it is still possible that prolactin has direct effects on the ovary, depending on tissue levels and the time of the cycle.

<u>Progesterone and symptom groupings</u>

Most authors studying hormonal parameters in PMS have not specifically stated their selective criteria for the symptoms. Bäckström et al (1974) studied women suffering from anxiety, irritability and headaches, but the diagnostic criteria were not defined, nor were they defined in a later paper (Bäckström et al., 1976b). Smith (1975) selected women with depression as the main symptom. Although these two authors found similar results for progesterone, Bäckströms group

found raised oestradiol levels while Smith did not. Among many other explanations for this, it is just possible that anxiety and depression have different aetiologies.

The majority of studies have tended to group anxiety, depression and irritability together as 'emotional lability' (Morton, 1950) or as 'negative affect' (Moos, 1969). Indeed, there is evidence from population surveys of women that premenstrual depression and anxiety are closely correlated (Moos, 1968; Golub, 1976). Despite this, an attempt has been made to sub-divide patients into those with 'anxiety-tension', 'asthenia-depression', 'irritability-explosiveness' and feelings of swelling, which were all defined and diagnosed by established criteria by Bäckström & Mattsson (1975). They found a high association between all the symptoms, and a high correlation between oestrogen levels at day M-3 and anxiety, irritability and to a lesser extent depression. They found no correlation with progesterone levels at day M-3, but this is not surprising as Bäckström et al(1974) had already shown that a deficiency of progesterone in PMS had disappeared by day M-3. However this was a study on a very few women with slight to moderate symptoms with a small amount of data on each. One interesting point to emerge is that feelings of swelling do not correlate with either hormone levels or with anxiety, although there is an association with depression.

Accordingly we had sub-divided our group into those suffering chiefly from the water retention grouping of Moos (1968) and those complaining of negative affect. The symptom groupings of Moos were used because they have been frequently used and tested by

subsequent authors (see Moos, 1977, for review). The 'negative affect' group in our study had lower progesterone levels, and in our earlier group of patients (Group I) low progesterone levels tended to be associated with raised oestradiol at day M-3 (Fig. 3.3). Thus, although we were not able to repeat the work of Bäckström and Mattsson, it appears that a similar trend was emerging.

The difference in progesterone levels that we observed is interesting in view of the well controlled study of Cullberg (1972) who was studying PMS from a completely different approach. By 'latent profile analysis' which is a method of classifying patients according to their tendency to cluster in response patterns to the questionnaires, he identified a group of women complaining of depression, irritability and tiredness before any medication, and these he regarded as a rather 'pure' PMS group. This class was also striking in that the women reacted badly to oral contraceptives with increasing proportions of oestrogen, but improved on the highly progestogenic combinations. Another class of women, suffering chiefly from weight gain and breast tenderness premenstrually, seemed to react less consistently to the various hormonal combinations.

These two classes would correspond with the 'negative affect' and 'water retention' types of PMS seen in our study, and which appear to differ in their progesterone status. This suggests that the mood changes are strongly hormone dependent, while symptoms of water retention may depend on individual responses at the tissue level. However it should be pointed out that there was considerable overlap of all the symptoms, and this was also found by Cullberg.

Some further support for the concept of two aetiologies has come from the double blind study of the therapeutic effect of bromocryptine and bumetanid, a diuretic (Andersch, et al. 1978 a). They found that the former treatment relieved tension and pain in the

breasts and irritability while the latter relieved bloating. From this rather slender evidence they suggest that mood changes, with mastalgia, occur separately from bloating.

Psychiatric illness and PMS

We had anticipated that this group might include some women suffering from neurotic illness, and that the premenstrual symptoms might be closely linked with and even secondary to the psychological disturbance.

Coppen & Kessel (1963) found a close association between neuroticism and premenstrual tension, and Clare (1977) using the General Health Questionnaire, found that GHQ positive (i.e. stressed) women had an 87% incidence of some premenstrual complaints while only 33% of unstressed women (GHQ negative) complained of such symptoms. However this is not evidence of causality in either direction.

It was expected that the GHQ positive women in our group might be characterised by their symptoms or some other aspect of their history but this was not the case. Neither did the progesterone levels differentiate between the stressed and the unstressed. However, interestingly, although a case of hyperprolactinaemia occurred in the GHQ positive group the rest of this group had lower prolactin levels than the 'non-neurotic' group. This trend is perhaps in the opposite direction to that expected, although as prolactin values were not available in all the women it may be rather premature to base any conclusions on this. An additional factor is that the criterion of a positive GHQ is only an indication of recent psychiatric ill-health or stress, but does not give a more specific diagnosis (Goldberg, 1972). Therefore at present it seems that PMS cannot be

subdivided neatly into psychosomatic and endocrine disease, although further work may clarify this.

Golub (1976) showed that anxiety and depression are raised premenstrually but that the extent of these temporary symptoms does not approach clinical depressive or anxiety states. This is borne out in our study, where being 'premenstrual' at the time of answering did not lead automatically to a positive score.

These results, taken overall, suggest that premenstrual tension is a true cyclical state and is not a function of personal adjustment to other stresses. The psychological illness seen in our sample and indeed seen in any sample of women (Clare, 1977) may yet be associated with some aspect of the menstrual cycle, but further research is needed.

CHAPTER 5

Treatment of premenstrual syndrome with dydrogesterone in relation to the progesterone status of the patients.

1. PATIENTS (GROUP III)

Patient Protocol

These women were largely self-referred following another discussion of PMS in the press, and after clinical assessment 103 women gave their informed consent to taking part in a single-blind controlled trial of dydrogesterone therapy. Initially the women were asked about eight symptoms; depression, irritability, lethargy, swollen breasts, swollen abdomen, swollen fingers or ankles, headaches and premenstrual stomach ache, and they were asked to keep record of these throughout the six-month trial, by giving each symptom a daily score from 0 to 3 (0 is absent, 1 mild, 2 moderate, 3 severe). Morning temperatures were taken daily.

Four blood samples were taken during the cycle preceding treatment, on days 12, 18, 22 and 26 of a 28 day cycle, and extending or shortening these intervals to adjust for longer or shorter cycle lengths. The sampling was repeated four cycles later after one cycle of placebo and two of dydrogesterone 10 mgm. b.d. and during the third cycle of active therapy. The course of medication was from day 12 until day 26, adjusted as above for longer or shorter cycles.

Progesterone estimations were carried out on all the samples and a computer index of the progesterone peak was calculated for 63 cycles before therapy and in 39 cycles during therapy.

Characteristics of the patients

36 women withdrew during the six-month trial; 15 of these stopped due to medical problems unconnected to the trial, 19 defaulted before treatment, and two gave up due to adverse effects on active treatment.

The remaining 67 women were of mean age 36 ± 5.2 (SD) years, range 24 - 44 years, and the menstrual cycle length was 27.9 ± 2.4 (SD) days, range 21 - 35 days. The other details about this group were very similar to Group II. The only slight differenne was that 79% of Group III were parous, compared to 59% of Group II, and this difference was reflected in a rather higher history of onset of PMS at childbirth in Group III; 41%, compared to 25% of Group II.

2. PLASMA PROGESTERONE VALUES

The mean progesterone peak for this group, as judged by the computer model, was 41.3 ± 13.3 (SD) nmol/l(n = 63). Of the 66 women who had at least one blood test, 21 (30%) were judged to have 'low' progesterone curves in comparison with our control group (see Fig. 4.1). The 'low' progesterone group included two patients who had short luteal phases of 7 and 8 days and one patient with a 'defective' luteal phase (values of 15 nmol/l or less).

The luteal phase was slightly shorter in the women with low progesterone peaks; a mean of 11.8 ± 2.0 days (n = 17) compared to 13.6 ± 1.0 days (n = 40) in the women with normal progesterone, and this difference was statistically significant ($p < 0.01$). The 'low progesterone' group did not have symptoms for a longer time than the 'normal progesterone' group (a mean duration of 9 days in both groups) nor was there any indication that the symptoms were more severe, since 60% of both groups were rated as having 'severe' symptoms by the physician.

Progesterone values and Response to Therapy

The overall response of the patients, whether they were the same, better, or worse, was determined from the physicians

assessment. 47 patients (72%) reported improvement during the months of active treatment, although initial improvement on placebo was 44%. However 15 (23%) reported improvement on dydrogesterone alone while only 4 (6%) reported improvement only on placebo. Seven (10%) reported that they felt worse on dydrogesterone, while only one reported that she was worse in the initial placebo month.

The response of individual symptoms was obtained by counting symptom scores cycle-by-cycle on the individual diaries. The results are shown in Table 5.1. Improvement was assessed by comparing mean scores for each treatment with the symptom score in the cycle preceding treatment.

Table 5.1 Reponse of individual symptoms to treatment with dydrogesterone and placebo.

		IMPROVED (No.)			MADE WORSE (No.)	
Symptom	No. complaining	Initial placebo	Active	Second placebo	Active	Placebo
Depression	56	18	44**	14	1	0
Irritability	61	19	41**	12	0	0
Tiredness	57	17	26+	11	1	0
Swollen breasts	56	11	14	15	9	3
Swollen abdomen	51	12	26*	21	5	2

Active greater than initial placebo < 0.01*

< 0.001**

Active greater than 2nd placebo month only: < 0.01+

The mean progesterone indices of the 'responders' and 'non-responders' was compared. Responders were considered to be those who responded to dydrogesterone alone or dydrogesterone plus one course of placebo, and non-responders were those who did not improve on the courses of active therapy or who responded well to all the courses, including all placebo treatments.

The mean progesterone index for the 'responders' was 39.7 ± 13.0 (SD) nmol/l (n = 38) and the mean for the 'non-responders' was 46.2 ± 13.6 (SD) nmol/l (n = 25), but this difference was not statistically significant (Students 't' test).

The mean of the seven adverse responders already included in the 'non-responders' above was 46.3 ± 9.9 (SD) nmol/l.

The mean progesterone values for the individual days were grouped into two-day intervals for the responders and non-responders (Fig. 5.1). It appears that in the 'responders' the luteal peak of progesterone occurs earlier and then regresses more sharply, but none of the differences were statistically significant.

There were no other differences apparent between those who did or did not respond, except that the responders had originally complained slightly more frequently of depression and irritability.

Progesterone values while on active treatment

Four samples for progesterone were obtained in both cycle 1 (pre-treatment) and cycle 5 (dydrogesterone) in 34 patients and during cycle 1 and cycle 6 (placebo) in five patients. The mean values for the computer index are shown in Table 5.2.

Table 5.2 Progesterone indices compared between pre-treatment cycle and either dydrogesterone- or placebo-treated cycle.

		PROG. Index nmol/l (mean, SD)			
Treatment	No. cycles	Cycle A pre-. treatment	Cycle B treatment	B/A % (mean ± SE)	P
B = dydrogesterone	34	41.7±12.6	35.1±15.0	84±4.3	<0.01*
B = placebo	5	46.2±6.6	44.8±18.9	97±4.5	ns

* 't' test, paired means

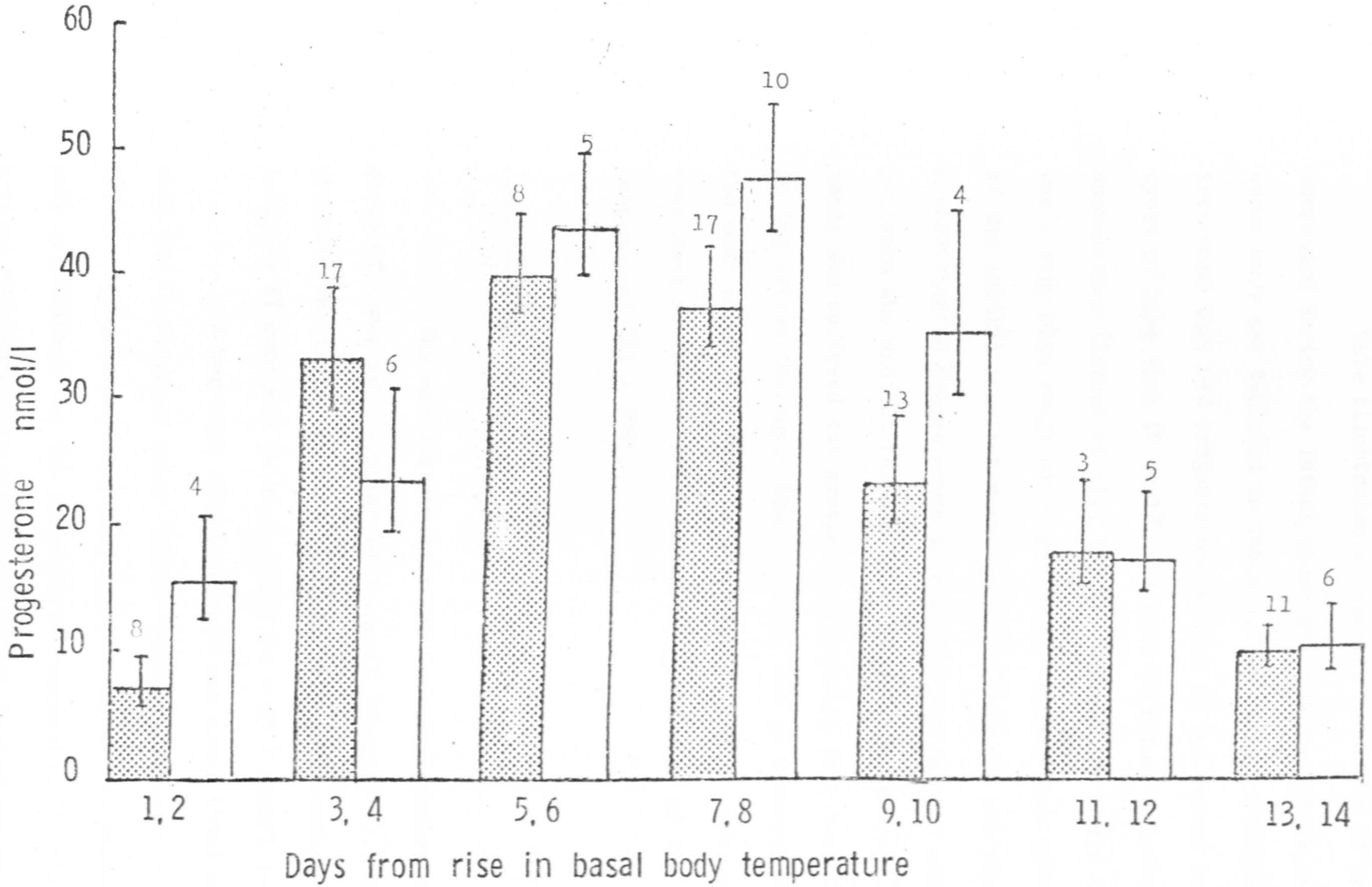


Figure 5.1. Log mean (± S.E.) plasma progesterone values at two-day intervals after the day of the rise in basal body temperature in women who responded to dydrogesterone(▒▒▒) and those who did not respond or were made worse(▭) Numbers at the top of the columns are the number of samples per two-day interval.

This illustrates an overall reduction of progesterone secretion during the luteal phase while under active treatment. Three women were not included in Table 5.2 as during dydrogesterone treatment they had progesterone values in the second half of the cycle of less than 10 nmol/l and were therefore considered to be anovulatory (Israel et al., 1972). Amongst the other women there was a very wide range of progesterone values, from 150% to 29% of the initial pre-treatment cycle, so that not everybody experienced a reduction in progesterone levels. It was thought possible that patients who did not respond to dydrogesterone therapy might be those who suffered the greatest suppression of luteal function during active therapy. However there was no difference between the mean percentage fall in progesterone during dydrogesterone treatment of the 20 'responders' (85 $\pm$ 26%) and of the 14 'non responders' (81 $\pm$ 27%).

3. DISCUSSION

The age distribution and menstrual cycles of the third group of women were similar to Group II (Chapter 4). The mean progesterone index was also similar; 38.9 $\pm$ 9.1 nmol/l (n = 77) in Group II compared to 41.3 $\pm$ 13.3 (n = 63) nmol/l in Group III.

A very high placebo effect was noted (44%) which agrees with the findings of other 'blind' studies (Mattsson & von Schoultz, 1974). It is interesting that a good response to dydrogesterone was only significant for the symptoms of depression, irritability and swollen abdomen and in some women breast tenderness and swollen abdomen were aggravated. The symptoms which improved such as

depression, are the most subjective ones where perhaps an 'improvement' could be suggested unconsciously by the physician, who was not 'blind' to the sequence of tablets. This pitfall was avoided as the individual symptoms were assessed from the daily scores on the patients' diary.

The two placebo effects, both before and after active treatment were very similar for most symptoms apart from swollen abdomen (Table 5.2) which probably rules out any 'carry over' effect of dydrogesterone.

The responders had slightly lower progesterone values than the non-responders although the difference was not sufficiently great as to suggest the role of dydrogesterone as hormone replacement therapy. One explanation for the difference is that the responders are a slightly different group, with more symptoms of depression, irritability and swollen abdomen these being the symptoms most responsive to treatment.

In fact the 'responders' in Group III did complain initially of slightly more depression and irritability than the 'non-responders'. This would also correlate with evidence suggesting that the women suffering from'negative effect' tend to have lower progesterone values (Chapter 4). There is no reason to believe that dydrogesterone and its main metabolite, DHD, interfere in the assay since the plasma levels of these substances in blood are thought to be in the low nanogram range (L.C. Post, personal communication), and the cross-reactivities of these substances was in the order of 1% for our antiserum (see page 88).

Indeed progesterone levels were low during the dydrogesterone-treated months. This could be due to some unknown

interference in the assay but it seems more likely that progesterone secretion is being reduced. Johansson (1971) administered progestogens to seven volunteers at three days after ovulation for varying lengths of time. He found that norethisterone and norgestrel, even at doses of 5 mg per day over six days or 100 mg per day over two days, reduced the progesterone levels over the remainder of the luteal phase to between 7 and 75% of the control cycles, and 300 mg clormadinone acetate and 360 mg medroxyprogesterone had the same effect.

In our study a total of 240 mg of dydrogesterone over 12 days gave a change in progesterone levels ranging from 150% to 29% of the control cycles, indicating that it is not as potent as other progestogens in this respect. The effect of dydrogesterone on luteal function has been noted before by Bell & Loraine (1965) although in their study the drug was given from day 5 to day 25, for dysmenorrhoea. In three out of five of their patients pregnanediol excretion was atypically low, and there was some doubt, from the excretory pattern of other hormones, whether two of these cycles were ovulatory.

The mechanism by which the progestogens reduce progesterone levels is not truly luteolytic, since a strong surge of progesterone can be induced by an injection of HCG (Johansson, 1971). There could be a negative feedback mechanism either on the hypothalamic-pituitary system or on the ovary itself. In support of the latter idea, there is some evidence that progestogens inhibit enzymes such as 3β-ol-hydroxysteroid dehydrogenase of the ovarian steroid pathway (Aakvaag, 1970) and the isomerases and aromatases (Schürenkamper & Lisse, 1978) (see Figure 6.1.)

Progestogens are not identical to progesterone in their metabolism, and their progestogenic properties may be combined with anabolic, oestrogenic and/or androgenic effects. The progestogens behave differently on the control of prolactin secretion (Gräf, Neumann, Nishino, Mehring & Hasan, 1973) and on the renin-aldosterone axis (Oelkers, Schöneshöfer & Blümel, 1974). Another complication is the suppression of endogenous progesterone levels, described above.

All these factors make it difficult to draw any conclusions about the role of progesterone in PMS from the success or otherwise of progestogen treatments. It is encouraging however that dydrogesterone is a significantly helpful treatment in PMS where other progestogens are not (Jordheim, 1972), and this may be partly due to its 'pure' progestogenic effects, that is without anabolic or oestrogenic qualities (Backer, 1962). However dydrogesterone had an apparently pharmacological and not a physiological therapeutic effect, and in addition may aggravate some of the symptoms.

In conclusion the results from Group III lend some support to the idea that progesterone activity may be involved in the emotional symptoms of PMS, although an absolute deficiency of progesterone is unlikely to be an important factor.

CHAPTER 6

General Discussion and Conclusions

General Considerations

There were a few general difficulties which are inherent in a study of this type; chiefly the classification and diagnosis of patients, the categorisation of symptoms and the collection of sufficient blood samples.

It was felt that a longitudinal self-report was a better diagnostic tool than a retrospective questionnaire such as that of Moos (1968). In as early as 1937, McCance, Luff & Widdowson found poor correspondence between these two types of record, and they concluded from their study of 167 women that little reliance could be placed on the use of a history or retrospective questionnaire. Also a longitudinal study yields more accurate information about the duration and timing of symptoms.

Another problem encountered was the variability of symptoms from cycle to cycle in each woman. Although the cyclical variation in subjective feelings is well documented, there is no evidence for a cyclic pattern to non-verbal behaviour which can be measured objectively, such as task performance (Sommer, 1973). Consequently there was no objective means to gauge how far outside factors might have affected a particular cycle. For instance, several women noted that the study cycle was atypical as they felt better than usual. This may have been a 'placebo' effect of the course of venepunctures, but we did not feel justified in excluding these cycles from the study.

It would have been far better to have daily blood samples from at least some of the patients, and although this was almost achieved with the controls and a few patients who were staff members, the other patients often came long distances and could attend for four samples at most. Other workers have partially resolved this

problem by daily sampling of only the last six or ten days of the cycle (Bäckström & Carstensen 1974; Bäckström et al., 1976b), although even this would have been difficult for most of our subjects.

Despite these difficulties we found some significant differences in endocrine relationships in the PMS group, and the individual findings have been discussed in Chapters 3 - 5. However it is possible to make some general conclusions from the project as a whole and to postulate an aetiological model from our findings. For this purpose the results in Chapter 3 regarding progesterone and oestradiol and those in Chapter 4 will be discussed.

The first finding of some interest is that progesterone levels are low in the early luteal and mid-luteal phase, but decline normally, while oestradiol levels are normal in the early and mid-luteal phases, but become increased four days premenstrually. The evidence for an imbalance between these two steroids is now quite strong, both from this work and that of others, as discussed in Chapter 3.

However, although a statistical difference was seen in Group 1, there were many women in Groups II and III with apparently normal or high progesterone levels. This implies that there are no sharply discrete 'pathological' disorders of steroid secretion in most women with PMS, but rather that the patterns of luteal function follow a continuous distribution. Indeed, examination of both columns in Figure 2.16 indicates that there is indeed a fairly continuous distribution of progesterone peaks, and the dividing line between 'low' and 'normal' progesterone is rather arbitrary. This is analogous to the continuous distribution of blood pressure values, with the consequent problems of deciding a dividing line for essential

hypertension (Taylor, 1977b).

Women with progesterone values at the extreme low end of the distribution curve might have the most severe symptoms (although we saw no evidence of this) or it could be that individual sensitivity is important. Another explanation could be that women with high or normal peaks of progesterone have their symptoms <u>after</u> the progesterone peak is reached, while the levels are falling rapidly, since at this time oestradiol levels are falling less sharply. If this were the case, one might expect women with high progesterone levels to have symptoms for a shorter time, but this did not emerge from the histories (see p. 197).

The alteration in the temporal pattern of steroid secretion during the luteal phase may be itself a causal factor in PMS, or it may be that a hypothalamic factor is the primary agent, inducing indirect changes in ovarian steroids. It may be pertinent in this context to discuss factors that are likely to control steroidogenesis in the corpus luteum, and which may therefore be responsible for the altered progesterone/oestradiol ratio.

<u>Possible mechanisms for the altered steroid ratio</u>

There are two possible ways in which an altered progesterone/oestradiol ratio could be brought about. The first mechanism could occur via alteration of the steroid pathways within the corpus luteum itself. The overall scheme for steroid biosynthesis in the ovary is shown in Figure 6.1.

Both granulosa and thecal cells from the Graafian follicle undergo luteinisation in the human, and both types of luteinised cell are found in the corpus luteum as a direct result of the presence of luteal thecal cells, since in species that secrete only progesterone

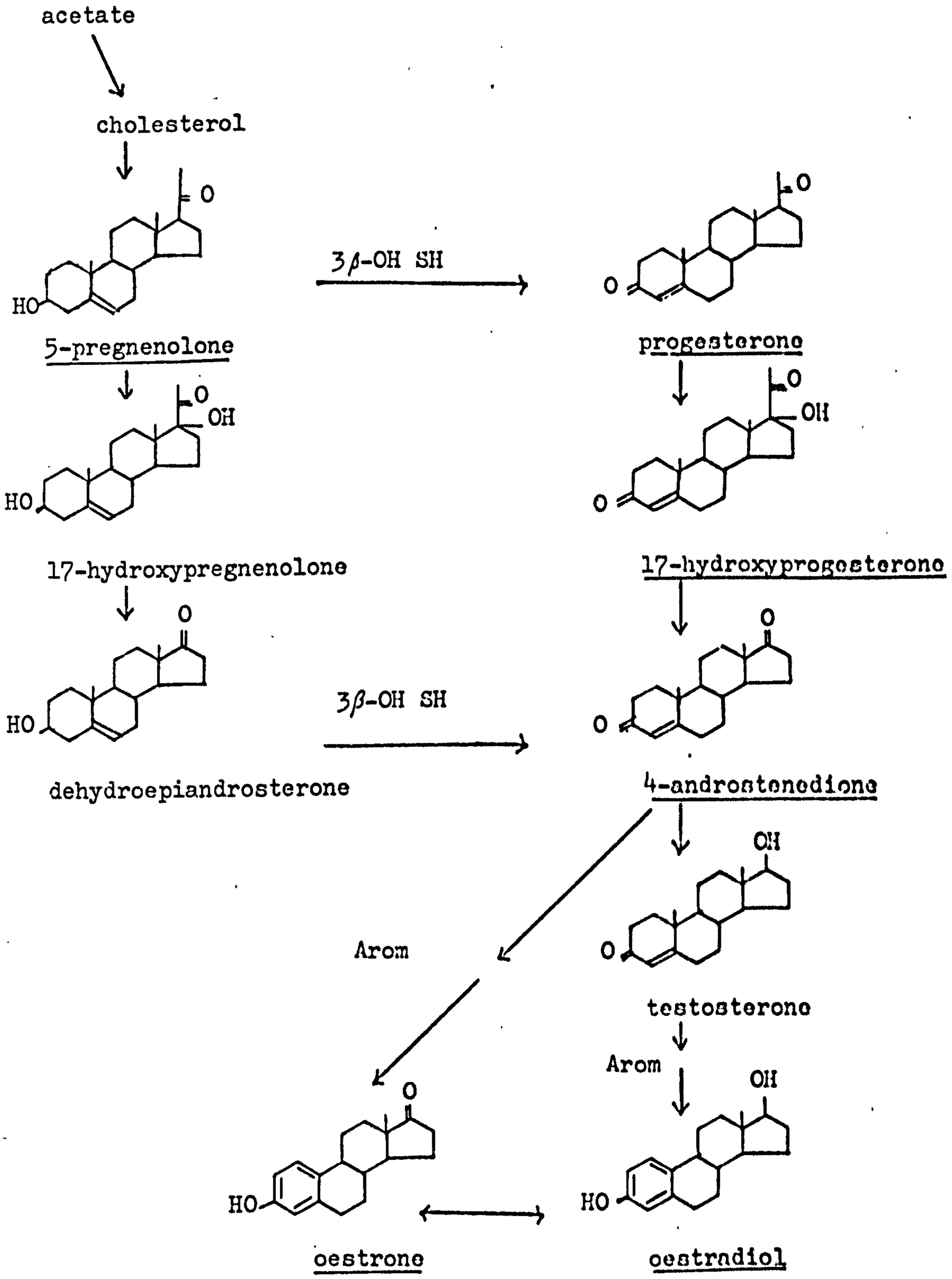


Figure 6.1. Steroid biosynthetic pathways in the human corpus luteum. The most important products are underlined. 3β-OH SH is 3β-hydroxy Steroid Dehydrogenase, and Arom is the enzyme aromatization complex.

after ovulation, such as the cow, only luteinised granulosa cells are present in the corpus luteum (see Eckstein, 1977, for review). It now seems established, from studies in vitro of carefully separated non-luteinised human cells (Moon, Tsang, Simpson & Armstrong, 1978; Fowler, Fox, Edwards, Walters & Steptoe 1978) that thecal cells produce the androgens, and these are aromatized by the granulosa cells.

Therefore relative numbers of thecal cells and granulosa cells could be a factor in the alteration of the progesterone/ oestradiol ratio, but control of these morphological changes is little understood, despite many histological studies.

It is possible that the gonadotrophins are able to alter the steroid balance within the corpus luteum, although the evidence so far does not support this. LH stimulates steroid synthesis by whole corpora lutea in vitro, but it stimulates all ovarian steroidsuniformly (Rice , Hammerstein & Savard, 1964), probably by control at the step from cholesterol to pregnenolone. It is possible however that FSH might have more subtle effects on the pathway. In rats FSH appears to stimulate aromatase activity of granulosa cells and also stimulates LH receptor sites (Dorrington, Moon & Armstrong, 1975). Although in humans the function of FSH after ovulation is uncertain, a role for FSH in subtle endocrine change seems possible, especially as Bäckström et al (1976b) found increased FSH levels in their PMS group in the early luteal phase (see below).

Another pituitary hormone, prolactin, may act directly on ovarian steroidogenesis. In rats prolactin is luteotrophic and the mechanism is possibly via its inhibitory effect on the activity of 20-hydroxy-steroid dehydrogenase, thus preventing the breakdown of progesterone to 20-hydroxyprogesterone (Armstrong, Knudsen & Miller, 1970). However the situation in the human corpus luteum is very

different, as prolactin has a biphasic stimulatory and then inhibitory effect on progesterone synthesis (McNatty et al., 1974), although it is not clear as yet whether prolactin affects oestradiol synthesis directly.

It appears from the above that there is no information at present about a single controlling factor which could cause the progesterone/oestradiol imbalance seen in our patients.

The second mechanism to account for the altered progesterone/oestradiol ratio has been suggested by Bäckström et al (1976b) and involves steroid synthesis by ovarian components other than the corpus luteum. These workers studied serum FSH and LH in the last ten days of the cycle in women with PMS, and they found that FSH was raised in these women compared to controls from 6 to 9 days premenstrually, although LH was unchanged. They also found that oestrogen was raised from day 5 premenstrually onwards, and they postulate that this reflects the stimulation of a second set of follicles, as a response to the increased FSH levels. They explain the subsequent decline in FSH levels in these women by negative feedback from the increasing oestrogen levels, so that the second set of follicles will regress at about the time of luteolysis. Presumably there is no positive feedback leading to further oestradiol secretion, an LH surge and ovulation in this second set due to the luteal levels of progesterone present, since Dierschke et al. (1973) found that high progesterone levels inhibit the ability of oestradiol to provoke positive discharge of LH.

Bäckströms group explain the increase in FSH levels early in the luteal phase as a response to the low levels of both progesterone

and oestradiol at this time, since the presence of low levels of both steroids lowers the threshold for FSH secretion (Bosu, Johansson & Gemzell, 1973; Hagino & Goldzieher, 1970).

What Bäckströms group do not account for, however, is the low steroid secretion seen by them in the early luteal phase, for both progesterone and oestradiol, which suggests some form of inadequate luteal development. We saw a slightly different pattern in that progesterone was low from 4 days after ovulation, but oestradiol was not markedly lower at this time (see Figure 3.4).

Two forms of inadequate luteal phase have been described in infertile, but regularly menstruating women; the short luteal phase, lasting less than ten days, with lowered secretion of both steroids; (Strott et al., 1970; Sherman & Korenman, 1974) and the defective luteal phase of normal length, but with lowered steroid secretion (Pearce, Fahmy, Morgan, Evans, Groom, Boyns & Cooke, 1971; Dodson MacNaughton & Coutts, 1975). The function of the corpus luteum in the women in our study resembled the latter, in that progesterone secretion was initially low in the luteal phase but the luteal phase was not markedly shortened. If this is the original defect in our patients, one might expect a proportion of them to be infertile, but although this information was not specifically sought, few women gave a history of infertility. However the age distribution was such that most women had completed their families and presumably secondary infertility would not be noticed. Furthermore anovulatory cycles, and therefore perhaps other cycle defects, are more common in older women (Sherman & Korenman, 1975).

Even if the defects seen in our study could be fitted into the existing categories of luteal phase defects, there is little known

about the aetiology of these defects which would help in the study of PMS. For instance, Pearce et al (1971) and Dodson et al (1975) found low pre-ovulatory levels of oestrogens and low levels of follicular phase FSH in the defective luteal phase but the LH peak was not altered. Unfortunately follicular phase levels of FSH were not measured by us or by Bäckström et al (1976b) but in our study the pre-ovulatory levels of oestradiol did not appear to be lowered (see Fig. 3.4).

Another type of defective luteal phase has been described by Lenton, Adams & Cooke (1978) in which only progesterone secretion seemed to be altered. A variety of treatments were applied, such as progesterone suppositories, anti-oestrogens and exogenous gonadotrophins, and although all these supplemented progesterone levels, infertility was not improved. These authors concluded that the underlying defect was not corrected. In an earlier paper Lenton, Sobowale & Cooke (1977) had noted slightly raised prolactin levels in the early follicular phase of women whom they suspected, but had not proved to have defective luteal phases. Prolactin has been implicated in infertility and PMS, as described previously, and high levels have been demonstrated in PMS patients compared to controls (Halbreich et al, 1976). Therefore it seems possible that prolactin hypersecretion may be one of the initial abnormalities in PMS, especially as an inverse relationship between prolactin and progesterone secretion in PMS has already been noted in Chapter 4 .

However interactions of prolactin with hormones of the menstrual cycle are little understood and rather controversial. Prolactin is known to inhibit the positive feedback of oestradiol on the LH peak at midcycle (see page 189) yet the mid-cycle LH peak

is unchanged in defective luteal phases (see above) and LH is unchanged in the luteal phase in PMS (Bäckström et al., 1976b). Other possible modes of action of prolactin are by increasing the refractoriness of the ovary to gonadotrophins (Rolland, Loquin, Schellekens & de Jong, 1976) or by depletion of LRF (Bohnet et al, 1975). Apparently the negative feedback of oestradiol is unimpaired in hyperprolactinaemia, from the evidence of McGarrigle et al (1978) discussed on page 189.

Another problem is that many studies of hyperprolactinaemia and the menstrual cycle have based their conclusion on studies of bromocryptine therapy, yet bromocryptine is effective in infertility even when prolactin levels are low (van der Steeg & Coelingh Bennink, 1977). This is attributed to the wider dopaminergic activity of bromocryptine acting directly on LRF or gonadotrophins. It could be therefore that raised prolactin is acting as a marker of reduced dopaminergic activity rather than affecting the cycle directly and this is discussed more fully on page 223.

Therefore, if prolactin is a factor in the endocrine changes seen in the premenstrual syndrome, its exact role is not known, although one can speculate that high levels of prolactin at some point in the follicular, early luteal, or even in the preceding luteal phase, are reflected in reduced steroid secretion in the early luteal phase, brought about either directly or via releasing factors or gonadotrophins.

The overall hypothesis is that transient high prolactin levels, due to stress, or a hypothalamic factor, lead to impaired follicular secretion of oestrogens or gonadotrophins and a 'defective luteal phase', followed by asynchronous follicular development as

described above. A summary of the postulated scheme is described in Figure 6.2. This hypothesis is attractive in that it explains the separation in time between the changes in progesterone and those in oestradiol. Also in this scheme of events one could envisage a 'vicious circle' being set up, whereby PMS-induced stress could trigger off events in the next cycle, and so on. This might explain the very high placebo effect in PMS.

There is also support for the scheme in that many different follicles are present in the human ovary at different stages of development, and that most of these follicles are destined for atresia despite early hormonal activity (Fowler, Chan, Walters, Edwards & Steptoe, 1977). Another possibility is that ovarian stromal tissue might make a steroidal contribution, stimulated by FSH in the luteal phase, although the principal products of stromal tissue in vitro are the androgens (Rice & Savard, 1966).

Although we can speculate that the scheme in Figure 6.2 is an explanation of the hormonal changes seen in our patients in Group I and Group II, it does not tell us which, if any, of these stages are responsible for the actual symptoms of PMS. Possible mechanisms will be described in the light of our results. As we obtained slightly different progesterone patterns for patients with chiefly psychological and somatic disorders, these components will be discussed separately.

HORMONAL CHANGES	STAGE OF CYCLE (M=last day menses)
Increased prolactin (or dopaminergic mechanism)	follicular and early luteal
refractory ovary → low oestradiol secretion	
decreased + ve feedback oestradiol → lowered LRF	
lower FSH and/or LH levels *	
poor corpus luteum (low progesterone, low oestradiol)	M-10 to -7
*	
increased FSH	M-9 to -6
stimulation of oestrogen in other follicles or stroma	M-4 to MO
anxiety, stress	
decreased dopamine *	late luteal
raised prolactin	late luteal, follicular

Figure 6.2 Possible mechanisms to account for changes in progesterone and oestradiol seen in our PMS study. Evidence for some of the stages (marked*) is described in the text.

The relationship between ovarian steroids and mood disorder

As mentioned in Chapter 1, there is evidence from work in animals that ovarian steroids accumulate in the brain and that the ovarian steroids influence behaviour. Although the conclusions from these animal studies cannot be extrapolated directly to humans, it now appears that there is a cycle to female-initiated sexual activity (Adams et al., 1978), and the effects of pharmacological doses of progesterone and oestradiol on human behaviour have already been mentioned (see Chapter 1, 3.1). These latter studies give the general impression that progesterone is tranquillising and hypnotic, while oestrogens tend to increase nervous activity. Seen in these general terms, it would appear that the tension and irritability seen in PMS patients in the last days of the cycle could be explained by the coincident lowering of the progesterone/oestradiol ratio.

However there are several observations which at first sight contradict this view. The first is that, as pointed out by Smith (1975), the progesterone/oestradiol ratio is far lower at the time of follicular ripening than at any time in the luteal phase and one would therefore expect 'pre-ovulatory' as well as 'premenstrual' tension. Such cases have been noted; Geiringer (1951) describes a woman with clear bouts of pre-ovulatory and premenstrual depression, lasting 2 and 4 days respectively, and Greene & Dalton (1953) also mention some cases with bimensual attacks, although no details are given. However in a prospective study daily scores of anxiety and aggression were lowest at midcycle, and the only peaks occurring at this time were a small increase in pain (presumably mittelschmertz), and peaks for 'self-rated pleasantness' and sexual arousal (Moos et al, 1969). Although

this was a study of only 15 women, some of them with PMS, an earlier study had noted the same effect (Altmann et al., 1941).

Examination of the daily charts of our patients was carried out in the hope of solving this problem. In Group I there was no indication of a bimensual pattern in the group as a whole (Fig. 3.1), although three individuals had bouts of irritability from 10 to 12 days premenstrually and again from 5 days before menses, and two women had a bout of depression between 18 and 14 days premenstrually, which could be interpreted as pre-ovulatory. There was no such pattern in breast tenderness or lethargy, and most of the women had no symptoms before the last week of the cycle. The same pattern was confirmed in Group III, where 10% of the women had a few days of irritability at around midcycle. Therefore it appears that pre-ovulatory tension is not a common feature of the syndrome.

One explanation of this could be that progesterone(or some other product of the corpus luteum) is synergistic with oestradiol at critical ratios, and such effects on behaviour are now established (Feder & Marrone, 1977). In ovariectomised rhesus monkeys administration of oestrogen leads to increased sexual activity towards the mate and aggression towards surrounding objects. If progesterone is added aggression towards the mate increases, (akin to the 'post-ovulation refractoriness' seen in rodents) but hostility decreases towards a third individual or inanimate objects (Zumpe & Michael, 1970). This has an interesting parallel in the subjective feelings of some of the patients in our study, who remarked that they were hostile towards their husbands and sometimes children, but not towards strangers or acquaintances, although family relationships were harmonic at other times of the month. A similar anecdote is mentioned by Dalton (1969).

A fascinating, but admittedly unlikely corollary to this is that married women have much higher depression rates than single women, although the opposite trend occurs for men (Weissman & Klerman, 1977), and it could be that 'hormone-induced hostility' to the mate, on a par with the work in monkeys of Zumpe & Michael (1970) may be a factor in marriage-induced depression. However it is more likely to have a psychosocial interpretation, in that married women tend to have low socioeconomic status, or it could even have a physiological interpretation, if childbirth is a precipitating factor in some forms of depression.

The second possible paradox for the 'imbalance' theory is that the menopause is often associated with depression, amongst other problems, yet oestrogens are very low at the menopause. However it now seems likely that there is no direct relationship between depression and the menopause (McKinlay & Jefferys, 1974) and preliminary double-blind trials on oestrogens in the menopause have proved ineffective for depression per se (Thomson, 1976), although other conditions such as bone-loss and hot flushes are certainly responsive.

One anomalous finding is in a double-blind trial of oestrogen by Klaiber et al (1974) for the treatment of depression of both menopausal and pre-menopausal women, where they found that oestrogens caused an improvement in mood, and this coincided with a decrease in plasma MAO activity.

Another slightly controversial point is the relationship between PMS and anovulatory cycles especially as both phenomena almost definitely coincide (Adamopoulos et al, 1972 ; Andersen et al, 1977).In Group III, three patients were anovulatory during dydrogesterone treatment and two of these recorded complete relief

during these cycles. However the confounding effect of dydrogesterone therapy makes these cycles rather hard to interpret, and as a few cases of anovulatory patients complaining of PMS have been noted in the clinic but were excluded from the studies, it seems likely that PMS does occur in anovulatory cycles. This may still be compatible with the 'imbalance' theory, since the progesterone/oestradiol is very low in the second half of anovulatory cycles (Sundsfjord & Aakvaag, 1972).

Assuming meanwhile that an ovarian steroid imbalance is at least partly responsible for the mood changes in PMS, it is still difficult to envisage the mechanisms at brain level. From work in animals and from studies of monoamine oxidase in the menstrual cycle reviewed in Chapter 1 it seems likely that the biogenic amines are mediating factors. The two main groups of biogenic amines are the indoleamines such as 5-hydroxytryptamine (serotonin) which is involved in serotonergic transmitter systems, and the catecholamines such as dopamine and noradrenaline which are involved in cholinergic and noradrenergic systems respectively. The metabolic pathways are shown in Figure 6.3.

Unfortunately we were not able to throw any light on the possible involvement of these systems from the results obtained during this project. But some information is available on the psychoendocrinology of some psychiatric disorders, and this may have links with the endocrinological results we have obtained. The only problem is that PMS has not been classified within the diagnostic framework of psychiatry. Most of the studies of psychoendocrinology have been on endogenous depression, and the relationship between this entity and PMS is uncertain at most, as PMS can be compared in magnitude to a moderate anxiety state (Golub, 1976) rather than to endogenous depression. Indeed in our work (in Group II) a measure

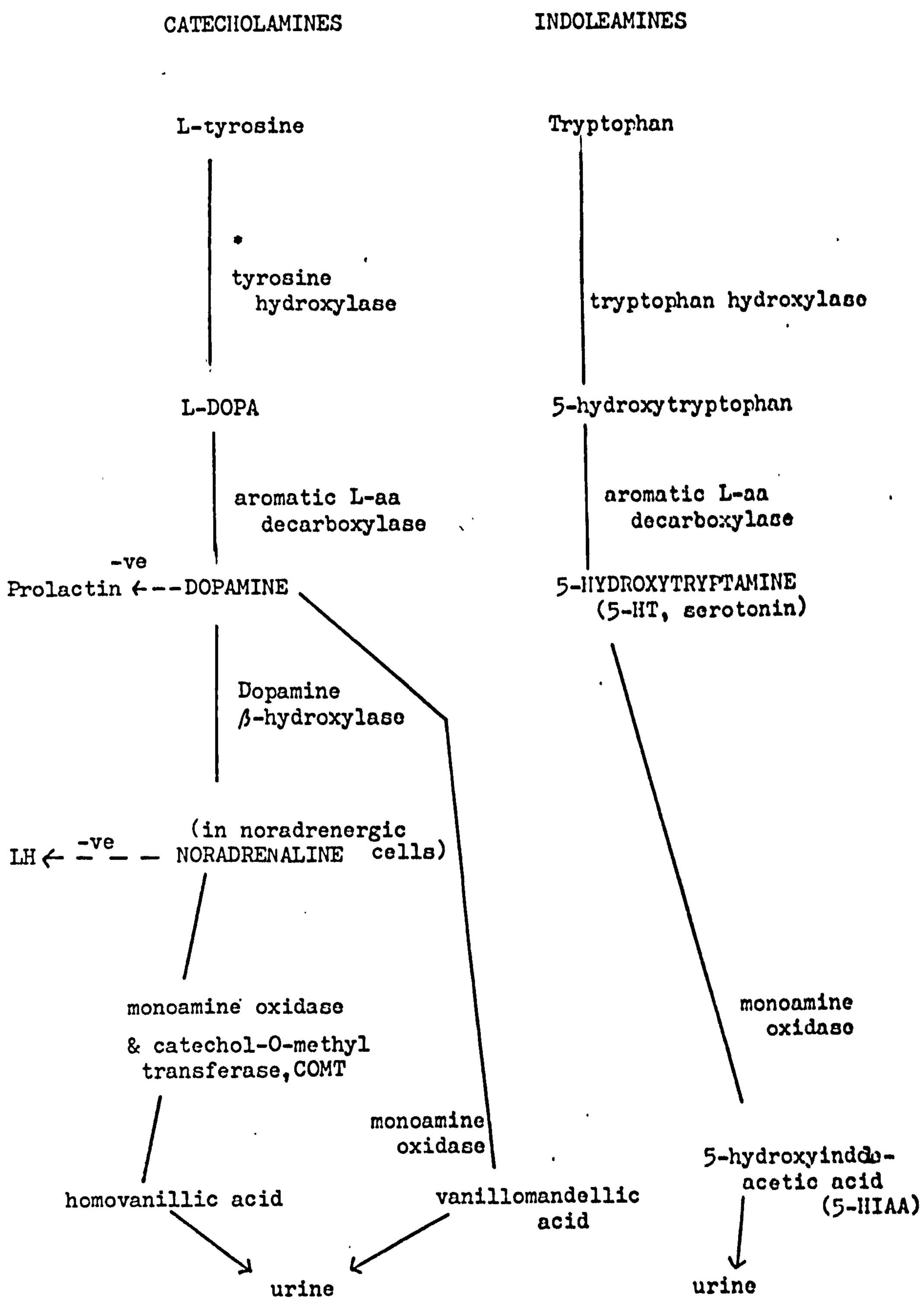


Figure 6.3. Metabolic pathways of the major biogenic amines in brain. Possible rate limiting steps (*) and also interactions with pituitary hormones are indicated.

of psychiatric ill-health did not appear to correlate with any particular aspect of PMS.

In the last decade a link has been established between neurotransmitter research and endocrinology when it was found that monoamine pathways are involved in the regulation of hypothalamic releasing factors. This is particularly encouraging as releasing factors can now be measured in blood (see page 14) and are therefore potential peripheral 'markers' of brain neurochemistry.

Most work has been carried out on the noradrenergic system. It was first noted, by Sachar, Hellman, Roffwarg, Halpern, Fukushima, Gallagher & Bronx (1973) that the 24-hour profiles of cortisol showed abnormally high peaks in patients with a particular type of endogenous depression. This condition did not seem to be a stress-related artefact, since the pattern was remarkably constant within and between individuals, and it returned to normal when the patient recovered. It was concluded that in certain types of depression there is a failure of the normal inhibitory influence of the brain on the release of ACTH and cortisol.

The neuronal control of CRF (corticosteroid releasing factor) and ACTH is complicated and encompasses serotonergic, noradrenergic and cholinergic systems, but there is some evidence, reviewed by van Praag (1978) that noradrenergic systems have an inhibitory effect on CRF, and it is this mechanism which is disturbed in patients with endogenous depression. For instance the response of Growth Hormone (GH) to insulin is disturbed in such depressed patients, and this mechanism is known to involve noradrenergic pathways. Thus cortisol appears to be acting as a peripheral marker of lowered noradrenaline function in depression. The gonadotrophins may also emerge as marks of central monoaminergic

activity. In a controlled study of depressed women in the menopause LH levels were lower in the depressed than in the non-depressed (Altman, Sachar, Gruen, Halpern & Eto, 1976). There is some evidence that LH secretion is mediated by noradrenaline (Ojeda & McCann, 1974), and assuming that oestradiol levels were the same in non-depressed and depressed groups (which was not tested), a deficiency of LH implies a defect in noradrenergic pathways.

The effect of dopaminergic systems in psychiatric illness is not yet certain although prolactin may emerge as a useful marker for central dopaminergic activity (Horrobin et al, 1976). Prolactin was raised in the last few days of the cycle in our patients, and this suggests a relative deficiency of dopamine at that time, although further work is needed.

Serotonergic effects in neuroendocrinology are also little understood, although serotonin (5-hydroxytryptamine) has been studied indirectly in depression by measuring levels of the precursor tryptophan in plasma, and metabolites in urine and cerebrospinal fluid. Levels of plasma free tryptophan have been inversely correlated with depression (Coppen & Wood, 1978), but the effectiveness of tryptophan treatment in depression is still disputed (Carroll, Mowbray, & Davis, 1970).

Much remains to be learnt about the effects of central amines on hormonal secretion and mood disorder, and especially the precise mechanisms involved. But the idea of a central noradrenergic defect in certain types of depression is supported by several neuroendocrine findings and this field is likely to expand in the future.

A second approach to neuroendocrinology is to study the

inverse relationships, that is the effects of hormones on neuro-chemistry and therefore ultimately on behaviour. Most of the work has of necessity been carried out in animals and some aspects are described in Chapter 1 (page 44).

There are many possible sites of controls of monoamine function, such as via the enzymes which synthesise and degrade the amines, or through the control of amine storage by the pre-synaptic nerve ending, or by affecting sensitivity of the post-synaptic receptors.

It appears that oestradiol decreases the activity of monoamine oxidase and decreases choline acetylase in rat hypothalami (Kobayashi, Kobayashi, Kato & Minkaguchi, 1966) . In women Klaiber et al (1974) found that oestrogens decrease plasma monoamine oxidase activity, while progesterone partially counteracts this effect.

Oestrogen metabolites also appear to control enzymic functions. Major metabolites of oestradiol in hypothalamic tissues are the extremely labile (2-hydroxylated) catechol-oestrogens (Fishman & Norton, 1975), and these are excellent competitive inhibitors of liver catechol-O-methyl transferase (COMT) the enzyme which degrades catecholamines (Ball, Knuppen, Haupt & Breuer, 1972). But this enzyme is unlikely to be rate-limiting (Ball et al., 1972), at least in peripheral conversions, and the functional significance of the other enzymic effects described above is still unknown.

La Torre (1974), from a preliminary study of the effect of oestradiol on eating behaviour, which is known to be affected by adrenaline, has argued that oestrogens prevent the conversion of dopamine to adrenaline. The relative concentrations of these monoamines may be important in behaviour as well as in gonadotrophin

control, especially in view of the evidence for the involvement of noradrenaline in depression (page 222).

Oestrogens may also increase brain serotonin levels indirectly by making available more of the free (presumably active) precursor, tryptophan, by competitive displacement from plasma binding proteins (Thomson, Maddock, Aylward & Oswald, 1977)

There is also evidence from in vitro studies in rat brain that oestradiol inhibits uptake of dopamine from thalamus, and progesterone and oestradiol inhibit uptake by cortex, pre-optic and septal regions, which is presumably an effect on receptor sensitivity (Wirz-Justice, Hackmann & Lichtsteiner, 1974).

It can be concluded that the oestrogens have wide-ranging effects on neurochemistry, but that the consequences of all the effects are probably not unidirectional. Although the very early 'amine hypothesis' implied that monoamines were deficient in depression (Schildkraut, 1965) this view has been modified in recent years and the role of the many components of amine function is still under debate (see page 39); therefore with present knowledge the exact role, if any, of oestrogens in mood disorder is still unknown.

The role of progesterone in neurotransmitter chemistry is even more obscure. The effects of progesterone on E.E.G. patterns are well known (Sawyer & Kawakami, 1961), and progesterone can also affect the uptake of serotonin, in vitro (Wirz-Justice et al, 1974); both of these effects could be due to an alteration in the sensitivity of post-synaptic receptors.

Some other interesting compounds are the saturated -3,20-oxygenated steroids which are very potent anaesthetics in mice (Holzbauer, 1976) and which are the chief metabolites of progesterone in rat hypothalami (Karavolas & Nuti, 1976). Those

compounds have no conventional progestational activity, but as they are located in neural tissue this strongly suggests that they are involved in neuroendocrine feedback mechanisms. 5α-pregnane-3,20-dione has a cyclical pattern in the peripheral blood of women, with a constant ratio of progesterone to pregnanedione of about seven (Milewich, Gomez-Sanchez, Crowley, Porter, Madden & MacDonald, 1977). It would be interesting to find the value of this ratio in women with PMS.

It seems therefore that very few conclusions can be drawn about the mechanisms at the basis of mood disorder, although some fairly new research would indicate that steroid patterns such as the 24 hour profile of cortisol are disturbed, in some forms of depression, but that they are acting as 'markers' of disturbances in releasing factor and hence neurotransmitter activity. Therefore the possibility exists that the progesterone/oestradiol ratio has no direct role in mood disorder, but is itself acting as a marker of disturbances at the hypothalamus or other higher centres. However the timing of the 'imbalance' (almost coincident with mood changes) and the evidence quoted above for the effects of progesterone and oestradiol on amine function and behaviour would suggest that the ovarian steroids have a more direct role in the production of mood change. The search for interactions between hormones and nerves at the biochemical level is only just beginning and should yield more answers in the future.

Possible mechanisms for the somatic symptoms

In Chapter 4 symptoms were grouped according to the clusters of Moos et al (1969) which groups breast tenderness and swelling together with symptoms of water retention. There was some indication, however that these two symptoms may behave differently. Although in Group III 41 women had both breast and abdominal swelling,

ten and twelve respectively had one symptom only. Dydrogesterone improved symptoms of swollen abdomen but in some women it made breast swelling worse (Table 5.1). Although this may reflect paradoxical effects of the progestogen on different target tissues, it also suggest different aetiologies.

Preece, Richards, Owen & Hughes (1975) measured total body water and weight in 17 controls and 39 women with mastalgia (defined as premenstrual breast pain with or without nodules) and they could find no association between water retention and breast symptoms. However this is not surprising as even where bloatedness is a main complaint in PMS, weight gain is not a feature (Bruce & Russell, 1962).

The other evidence for treating breast swelling and bloatedness as separate complaints is that Andersch et al (1978a) found that mastalgia responded to bromocryptine, while bloatedness was relieved by a diuretic. Therefore the aetiology of these two symptoms will be considered separately.

The study of breast pathology has centred on neoplastic conditions and there is curiously little information about benign disease. However in vitro studies have established that human mammary development is under complex control with the involvement of prolactin, growth hormone, insulin, adrenal and ovarian steroids (Ceriani, Contesso & Nataf, 1972).

Sitruk-Ware et al (1977) have found low progesterone levels but unchanged oestradiol levels in women with cyclical and non-cyclical breast disease, which was a mixed group defined as mastodynia with or without cystic mastitis. It was shown in Chapter 4 that low progesterone is associated with slightly raised prolactin and the association of high prolactin levels with breast disease is well known. Indeed bromocryptine is effective in

reducing premenstrual breast discomfort (Andersen et al., 1977, Andersch et al., 1978b). All these facts point to a high prolactin (and consequent low progesterone) as responsible for breast symptoms.

Yet this idea is at variance with our finding of high or normal progesterone in women complaining chiefly of the admittedly heterogeneous water retention cluster, which includes breast tenderness, and also with the adverse effects of dydrogesterone on this symptom (Chapter 5). There may be other explanations, for instance it could be that the prolactin/oestrogen ratio is the critical factor.

It has been suggested that in the puerperium milk production is a response to rapid falls in oestradiol after birth combined with unchanged levels of prolactin (McNeilly, 1975). This suggests a prolactin/oestrogen antagonism on the breast, and indeed breast tissue is most sensitive to stimuli after ovulation and at menstruation, when oestradiol levels are falling (Robinson & Short, 1977). This biphasic pattern is not seen however in the breast symptoms of the patients, although in some women pain and swelling starts 14 days premenstrually (Fig. 3.1). Another factor must be local since Preece, Hughes, Mansel, Baum, Bolton & Gravelle, (1976) in one of the few surveys of benign breast disease, found that even premenstrual breast pain might be confined to one breast or part of the breast. This could be due to hormonal effects on fibrocystic scars.

In this respect it is interesting that half our control group (Table 3.1) also had breast symptoms and in their survey of G.P. practices Kessel & Coppen (1963) found breast swelling in over half the women sampled. Before the aetiology can be defined a clear classification between physiological and non-physiological breast change is needed.

As already described in Chapter 1, consistent weight gain is not a feature of PMS, although Good (1978) has shown that

serum osmolality varies inversely with oestradiol in the normal menstrual cycle, and he has suggested that uptake of water by tissues may be a critical factor in events of the menstrual cycle, for instance water uptake by the follicular fluid may be the mechanism for ovulation. In target tissues such as endometrium hydration may occur in response to protein synthesis, but this seems an unlikely explanation for abdominal bloatedness. This symptom remains therefore somewhat of a puzzle, although local actions of oestrogen together with other hormones, remains the most likely explanation.

Further research into PMS

Although attracting some attention from psychologists and gynaecologists, PMS has been the subject of few properly controlled endocrinological studies. This is partly a result of technical difficulties of hormone assay in the past which are now largely overcome. Another difficulty has been the problem of defining the syndrome, especially in view of the 'overlap' between gynaecology and psychology.

However PMS merits further endocrine research for several reasons. The first is that, although not a life-threatening disorder, there is a very high degree of personal and family distress in severe cases. Secondly, mental illness in various forms takes up a very high proportion of hospital beds and also outpatient care in the National Health Service , and depressive illness has twice the incidence in women compared to men (Weissman & Klerman, 1977).

Although it has been repeatedly emphasised in this thesis that PMS does not bear any obvious relationship to classified mental illness, the fact that the sex disparity in depression rates is fairly constant in different cultures argues that it has some physiological basis. Therefore on economic grounds alone any research which casts light on at least some aspects of mental illness is to be welcomed, and PMS is a fascinating model in that each patients acts as her own control in having phases of 'illness' and 'recovery' in the same cycle.

Much remains still to be learnt about the hormonal changes in PMS. It may yet emerge that one variable, such as a steroid, hypothalamic or pituitary hormone, or neurotransmitter, may be grossly altered in the syndrome, but it seems more likely that more subtle endocrine changes will be found.

The most pressing need to emerge from this thesis is for a daily comparison of LH, FSH, progesterone, oestradiol and prolactin throughout more than one cycle in at least twenty PMS patients and twenty controls. This would help to clarify some of the controversy in the literature regarding the levels of oestrogen and progesterone in PMS, which may have been caused partly by small numbers of patients in previous studies. It would also help to confirm and extend the interesting observations of Bäckström et al (1976b) regarding gonadotrophin levels. Furthermore, although several papers have now looked at prolactin throughout the cycle, there is no agreement regarding a cyclical pattern. A daily study of both women with PMS and controls may help to clarify why these discrepancies exist.

However such a study would be of limited value without a proper definition of PMS and a universally agreed method

of assessing the syndrome. From our own work it appears that the mental and somatic symptoms can be distinguished in terms of response to therapy within the same individual and in terms of hormone patterns between groups of women. It seems therefore that further research will be more fruitful if these various components can be dissected out, but with due regard for the relationships between the, unlike some psychologists who seem to ignore the somatic symptoms completely (Parlee, 1973).

The symptom diaries in our study were useful, but valuable data about the type of mood change was often missed. The Moos Menstrual Distress Questionnaire also suffers drawbacks in that itmay induce over-reporting of symptoms (Ladisich, 1977) and the symptoms 'clusters' found by Moos (1968) are rather too general. Cullberg (1972) and Golub (1976) have tried other psychological approaches with some success, although they had to use a whole battery of different questionnaires.

A useful approach would be to measure 'trait' anxiety and depression in patients at the first clinic visit, along the lines of the study of Golub (1976). This would give a quantitative measure of the patients background mental state, and it could be combined with a short retrospective menstrual questionnaire. This could then be confirmed during the study by a daily record of symptoms, preferably on a tear-off pad so that the patient would immediately remove the record for each day, and so could not be influenced by the previous days reports.

At the same time, in this postulated study, it would be useful to have an objective gauge of bodily changes, such as oedema, which could be measured by change in ring size or abdominal girth. Feelings of breast swelling could be measured

by the water displacement technique used by Milligan, Drife & Short (1975).

Although we were not able to show an definite changes in aldosterone in our PMS group, the conditions were not as controlled as we would have liked, and relatively few patients were studied. Therefore a study of aldosterone, renin activity, and angiotensin II, all of which are known to have a cyclical pattern, would be most useful contribution. It would also be interesting to study the response to upright posture in women with PMS throughout the cycle, as it has been shown by Edwards & Bayliss (1973) that the retention of sodium on upright posture is increased in the luteal phase. It may be that this effect is exaggerated in women with premenstrual bloatedness.

Another hormone which may be of interest is testosterone. A female preponderance of depression, as described above, could have another interpretation in that some factor in men actually protects them from depression. Testosterone could be such a hormone, especially as there is a mid-cycle peak in testosterone levels (Judd & Yen, 1973) which coincides with the time of self-rated 'pleasantness' in women (Moos et al, 1969). It is possible that it is the interactions between testosterone and other hormones such as oestradiol which are important.

Several of our patients mentioned a craving for sweet things premenstrually, and a paper by Smith & Sauder (1969) suggests that this has a psychological element, but a physiological basis exists in some women. This craving, and a tendency to bruise easily, which was also mentioned by a few of our patients, is thought to be due to hypoglycaemia (Billig & Spaulding, 1947). Therefore it would be of interest to carry out glucose tolerance tests in some

women with PMS, and perhaps linking them with progesterone estimations, since as already described, progesterone is catabolic in man (unlike the situation in animals) and lowered amino-acid levels due to progesterone could stimulate appetite and sugar craving. It would also be interesting to study cortisol levels, since cortisol is required for gluconeogenesis, and especially as cortisol is one of the few steroids which seems to have a characteristic and genuine alteration in secretion with depression (see page 222). The ideal study would be 24-hour profiles of cortisol in women with PMS, during follicular and luteal phases.

The study of neurotransmitter biochemistry and the biogenic amines in particular, would be fascinating, but studies of substances such as monoamine oxidase in blood are the only practical measures at the moment. The measurement of the monoamines themselves is technically difficult and peripheral levels are probably misleading. One useful development however is the link between central amines and releasing factors such as luteinising hormone releasing hormone and prolactin inhibitory factor(dopamine).

There is some evidence that some of the releasing factors have behavioural effects (Griffiths, Hooper & Jeffcoate, 1975), and it may emerge that they are very closely linked indeed to neurotransmitter biochemistry. As some releasing factors are now measurable in peripheral blood (Arimura et al., 1974) they may yet prove to be useful peripheral markers of noradrenergic and serotonergic systems in the CNS. Prolactin may already be regarded as a 'marker' of cholinergic systems and a 24-hour profile of prolactin in PMS may show up important follicular and luteal differences.

The possibility of an animal model for PMS seems

rather more unlikely at the present time, but it would be extremely useful in the study of central mechanisms. It might be possible to study monkeys or primates with true menstrual cycles, but a measure of 'premenstrual tension' would have to be devised in these animals, and unfortunately there are no objective non-verbal measures of PMS as yet which could be applied to these animals.

Other hormones to measure could be the highly anaesthetic metabolites of progesterone, such as pregnanedione, which is present in large amounts in neuroendocrine tissue. It could be that this hormone is the important one in central affect, rather than progesterone itself, of which the main target organ is the uterus.

Another interesting compound is pyridoxine, which is a co-factor in key steps of the metabolic pathway of serotonin and also of dopamine. It could be that a deficiency of this vitamin occurs in some women or that some women have a greater requirement than others, perhaps influenced by oestrogen levels (Brush, 1977). Therefore a study of pyridoxal phosphate in plasma may help to discriminate still further between different forms of PMS.

There are also many studies which could be made outside the fields of endocrinology, but which may help in the understanding of this syndrome. So far the study of treatments has not been very helpful, as the studies have been uncontrolled or on few women, or because pharmacological agents have been used. For instance in our study of dydrogesterone, a fairly 'clean' progestogen, there were adverse effects on luteal function, and improvement seemed to be due to a pharmacological rather than a physiological action.

One useful study would however be a double-blind

trial of progesterone itself. Although this has problems as progesterone has to be given as either suppositories or by injection, progesterone is one of the few treatments where almost total success has been claimed (Dalton, 1977).

Another observation, by Benedek-Jaszmann & Hearn-Sturtevant (1976) was that PMS occurred in association with infertility. There were flaws in this study, but the findings have important implications for the understanding of both conditions. For this reason it would be interesting to study the incidence of PMS in women with unexplained infertility (that is, no obvious anatomical or endocrinological reasons) and to compare this with the incidence in a matched control group, for instance a group of parous women attending a family planning clinic.

It has also been suggested that individual predisposition plays a part in PMS, and some studies of the incidence in families, beyond anecdotal reports, may determine whether the disorder has any genetic component.

SUMMARY OF ORIGINAL FINDINGS

1. In a comparison of 20 women with premenstrual syndrome and 10 control cycles there were significantly lower progesterone values from 5 to 8 days premenstrually, and significantly higher oestradiol levels in the last four days of the cycle, in the women with PMS. This confirms and extends observations by Bäckström & Carstensen(1974) and Bäckström et al (1976b).

2. There were no significant differences in plasma aldosterone in PMS patients and controls under non-fasting, ambulant conditions. In both groups there was a gradual pre-ovulatory rise in plasma aldosterone levels and a second rise in mean

aldosterone values in the luteal phase, although levels were highly variable.

These findings confirm a cyclical pattern of secretion of aldosterone in the normal menstrual cycle, as found by several authors, but there was no evidence from this study that the renin-angiotensin-aldosterone system is altered in the premenstrual syndrome.

3. In a larger group of patients (105) the symptoms were classified and those complaining chiefly of depression and irritability had significantly lower progesterone plasma levels during the luteal phase than those patients complaining chiefly of breast tenderness and bloating as their main symptoms. Progesterone values of women not complaining of premenstrual symptoms were intermediate between the mean values for the two symptom groupings.

4. The incidence of psychiatric ill-health in the women was quite high (45%) but this ill-health could not be correlated with a particular pattern of progesterone values or any other characteristic of the patients studied.

5. Prolactin was also measured in the serum of some PMS patients and although not compared with a control group for PMS, the values were within the range for women without obvious pituitary disease.

Prolactin levels in the early luteal phase were higher in women with low progesterone peaks, which may suggest a relationship between prolactin and the ovary .

6. In a study of the therapeutic effect of dydrogesterone during the luteal phase , in a single-blind trial 47 women (72%) responded to active treatment but no significant differences could be seen in the progesterone levels of those women who responded compared to those who did not. Dydrogesterone lowered plasma progesterone levels to a mean of 84 % of pre-treatment levels, although anovulation occurred in only three cases.

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[Reprinted from the Proceedings of the Society for Endocrinology, 25–26 November 1976.]
JOURNAL OF ENDOCRINOLOGY, [1977], **73**, 21*P*

 PRINTED IN GREAT BRITAIN

Progesterone and aldosterone levels in the premenstrual tension syndrome. By Madeleine Munday, M. G. Brush and R. W. Taylor. *Department of Gynaecology, St Thomas's Hospital Medical School, London, SE1 7EH*

The premenstrual tension syndrome (PMT) is characterized by depression, irritability, personality change and swelling of abdomen and breasts during the 7–14 days before the onset of menstruation. Not all symptoms are present in all patients and some may appear later than others in the premenstrual phase. Little evidence is so far available on the endocrine status of these patients and, therefore, we have measured plasma progesterone and aldosterone levels in eight PMT patients aged 22–49 years (mean 35 ± 10) and in eight control patients aged 22–32 years (mean 27±3).

At least six blood samples were taken from each subject throughout each menstrual cycle and progesterone and aldosterone levels were measured in plasma using a radioimmunoassay. The approximate time of ovulation was determined by basal body temperature measurements. Posture was standardized by 30 min recumbency but diet and time of day of blood sampling were not controlled.

The menstrual cycles of the PMT patients were shorter than those of the controls (26·3 ± 1·1 days compared with 29·6 ± 1·6 days).

Progesterone values for the PMT patients for each day of the cycle (dated from the next menstrual period) did not differ significantly from those of controls except during the mid-luteal phase. At this time (5–8 days before menstruation) the mean plasma progesterone value was 31 ± 10 (S.E.M.) nmol/l for PMT patients and 45 ± 14 nmol/l for controls ($P = 0{\cdot}02$, using Student's *t*-test).

Plasma aldosterone values in the control group showed an increase in the preovulatory and early luteal phases, in agreement with the results of Frolich, Brand & van Hall (1976).

In PMT patients, the plasma aldosterone values were not consistently different from those of controls. There was a small surge in the late luteal phase of the PMT group but this was not statistically significant.

When the ratio of progesterone to aldosterone was estimated, it was found that this ratio was markedly lower in the PMT group in the mid-luteal phase only ($P = 0{\cdot}05$). This difference was probably due to the influence of progesterone values alone.

A large group (105) of PMT patients is now being studied to elucidate the steroid and prolactin profile in detail, with initial results giving further evidence for impaired progesterone levels in the luteal phase in some patients.

These findings can be compared with those of Backstrom & Carstensen (1974) who found a depression of plasma progesterone in ten PMT patients with marked anxiety at 4–6 days before menstruation. They also found higher plasma oestradiol levels at 2–5 days before menstruation.

The role of aldosterone in this condition is incompletely understood but clearly any excess aldosterone secretion would tend to promote the fluid retention often seen in PMT. Equally, it is known that progesterone tends to oppose aldosterone action and promote sodium loss (Landau & Lugibihl, 1961; Oelkers, Schoneshofer & Blumel, 1974). Prolactin may also be implicated (Halbreich, Assael, Ben-David & Bornstein, 1976) with higher serum levels being seen in some PMT patients. Studies are in progress to assess the fluctuation of prolactin and oestradiol in this syndrome.

We are indebted to the Endowment Fund of St Thomas's Hospital for a research grant.

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Current Medical Research and Opinion Vol. 4, Suppl. 4, 1977

Hormone levels in severe premenstrual tension

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Curr. Med. Res. Opin., (1977), 4, Suppl. 4, 16. Paper read: 29th January 1977

Summary

Plasma levels of progesterone and aldosterone during the menstrual cycle were studied in 8 patients with premenstrual tension and in 8 controls. The results showed a deficit in progesterone 8 days premenstrually in the women with severe symptoms. In 6 of the 8 patients in this first study, symptoms started before this, suggesting the involvement of some other factor earlier in the cycle. Aldosterone levels, however, showed no consistent change even in women with low progesterone levels, and there was no difference between patients who gained weight cyclically and those who did not. In a second ongoing study the levels of these hormones and of prolactin were followed in a larger group of 58 women with severe premenstrual symptoms. Raised prolactin and low progesterone levels were found in approximately 30% of the 58 patients during the second half of the cycle suggesting that prolactin reduces progesterone production in corpus luteum cells, and this slight deficiency was reflected in somewhat shorter length menstrual cycles. Further studies are being carried out to clarify the roles of prolactin and oestradiol in this syndrome.

Key words: Premenstrual tension – progesterone – aldosterone – prolactin

Introduction

Until recently, little work has been carried out to investigate the hormonal basis of the premenstrual tension syndrome. This was due, in part, to the lack of adequate laboratory methods to measure hormone blood levels. In the last few years, however, the technique of radioimmunoassay has made possible the study of very slight changes in the hormonal pattern.

Many theories have been put forward to account for premenstrual tension. In particular there has been a long standing belief, first suggested by Frank,[3] that an imbalance exists between the ovarian steroids, oestradiol and progesterone. This idea was supported by several authors such as Israel[3] who thought that the ratio of oestradiol to progesterone was excessive. A second idea for the aetiology of premenstrual tension was prompted by the finding[6] that aldosterone secretion rates were raised in the second half of the normal cycle. Several workers[10] have suggested that increased activity of the renin-angiotensin-aldosterone system might be involved, and this might account for the symptoms of water retention seen in this condition. Recently, a third idea was put forward by Horrobin.[8] He suggested that

prolactin might play a crucial role in this syndrome. He also pointed out that prolactin retains sodium, water and potassium and he reported that this also occurred in premenstrual tension. Prolactin, however, does not have a consistent cyclical pattern.[13]

There has been some evidence so far for all of these hypotheses, although some of the evidence is conflicting. It was decided, therefore, to study three steroids – progesterone, aldosterone, and oestradiol – in a small group of patients and later to study these hormones and prolactin in a larger number of women to see what variations there were in levels during the menstrual cycle and if there were any correlations with premenstrual symptoms. The study is still in progress and this paper is an interim report of the findings.

Methods and patients

The first group of women consisted of 8 with premenstrual tension who had been referred to the gynaecological clinic, and 8 controls from the hospital staff. During one cycle each patient and control completed a daily record of symptoms and attended for at least 6 blood samples. Diet and time of day were not controlled, although the patients remained recumbent for 30 minutes before blood sampling.

The second group of 58 women complained of severe premenstrual tension and were obtained via an appeal in the press. Their symptoms were much more variable than those of the first group. The patients also completed a daily record of symptoms, but on average only 1 blood sample was taken in the follicular phase and 3 samples in the luteal phase. Since it has been found recently that 30 minutes recumbency does not reduce aldosterone to basal levels,[15] patients in the second group were not recumbent during blood sampling. Progesterone was measured by radioimmunoassay using an antiserum to progesterone-11α-hemisuccinate-BSA, and conditions modified from those of Furr.[5] Aldosterone was measured by radioimmunoassay using an antiserum to aldosterone-18,21-dihemisuccinate-BSA, kindly supplied by the National Institutes of Health, U.S.A. Aldosterone was extracted with dichloromethane and purified by column chromatography on Sephadex LH20 (Pharmacia) before assay. The prolactin assays were carried out in the Chemical Pathology Department.

Results

Group 1

The pattern of cyclical symptoms experienced by the patients and controls is shown in Table I. The average duration of symptoms was 10 days. Out of the 6 patients who kept regular weight records, only 3 experienced weight gain of more than 2 pounds. It is also interesting that the symptom of breast tenderness was recorded by half the controls.

Table I. Pattern of cyclical symptoms experienced premenstrually by 8 women with severe premenstrual tension syndrome and by 8 controls

Symptom	Premenstrual tension patients	Control group
Depression	6	
Irritability	7	
Lethargy	5	
Breast tenderness	5	4
Abdominal oedema	7	1
Headache	2	
Weight gain over 2 lbs	3	

The ages and menstrual cycle lengths of the 16 women studied are given in Table II. Unfortunately, we could find only rather young control volunteers. Differences in mean menstrual cycle length and luteal phase length (judged by body basal temperature) were evident.

Table II. Age, length of cycle, and length of luteal phase in 8 women with severe premenstrual tension and in 8 controls: mean values (±S.D.)

Data	Premenstrual tension patients	Control group
Age (years)	35.0 ±10.0	27.1 ± 2.9
Length of menstrual cycle (days)	26.3 ± 1.1	29.6 ± 1.6
Length of luteal phase (days)	13.1 ± 1.7	14.5 ± 0.5

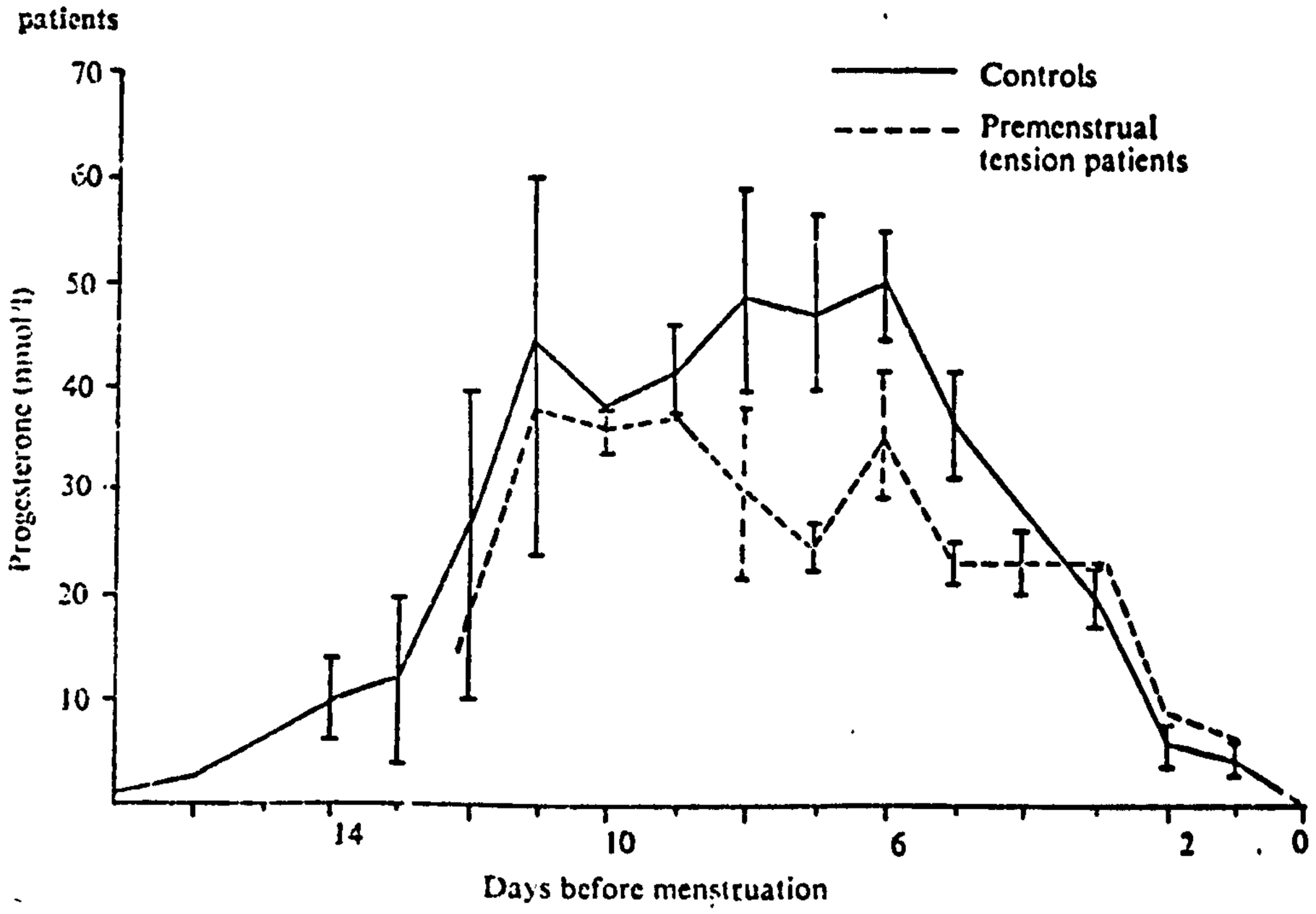
Shorter luteal phases have been shown to be unrelated to age.[16] It seems, therefore, that the premenstrual tension patients had shorter cycles and shorter luteal phases than the controls.

Plasma progesterone values for the patients and controls are given in Figure 1. All cycles in both groups showed a progesterone peak, indicating ovulation, but there were lower values for the premenstrual tension patients for the Days minus 8 to Days minus 5 period before menstruation.

These results are in agreement with those reported by Backstrom and Carstensen.[1] They studied the last 6 days of the cycle and found lower progesterone levels on Days minus 6 to Days minus 4 before menstruation in their 10 patients and controls. These results suggest that the ratio of progesterone to oestradiol is reduced at least in the last week of the cycle.

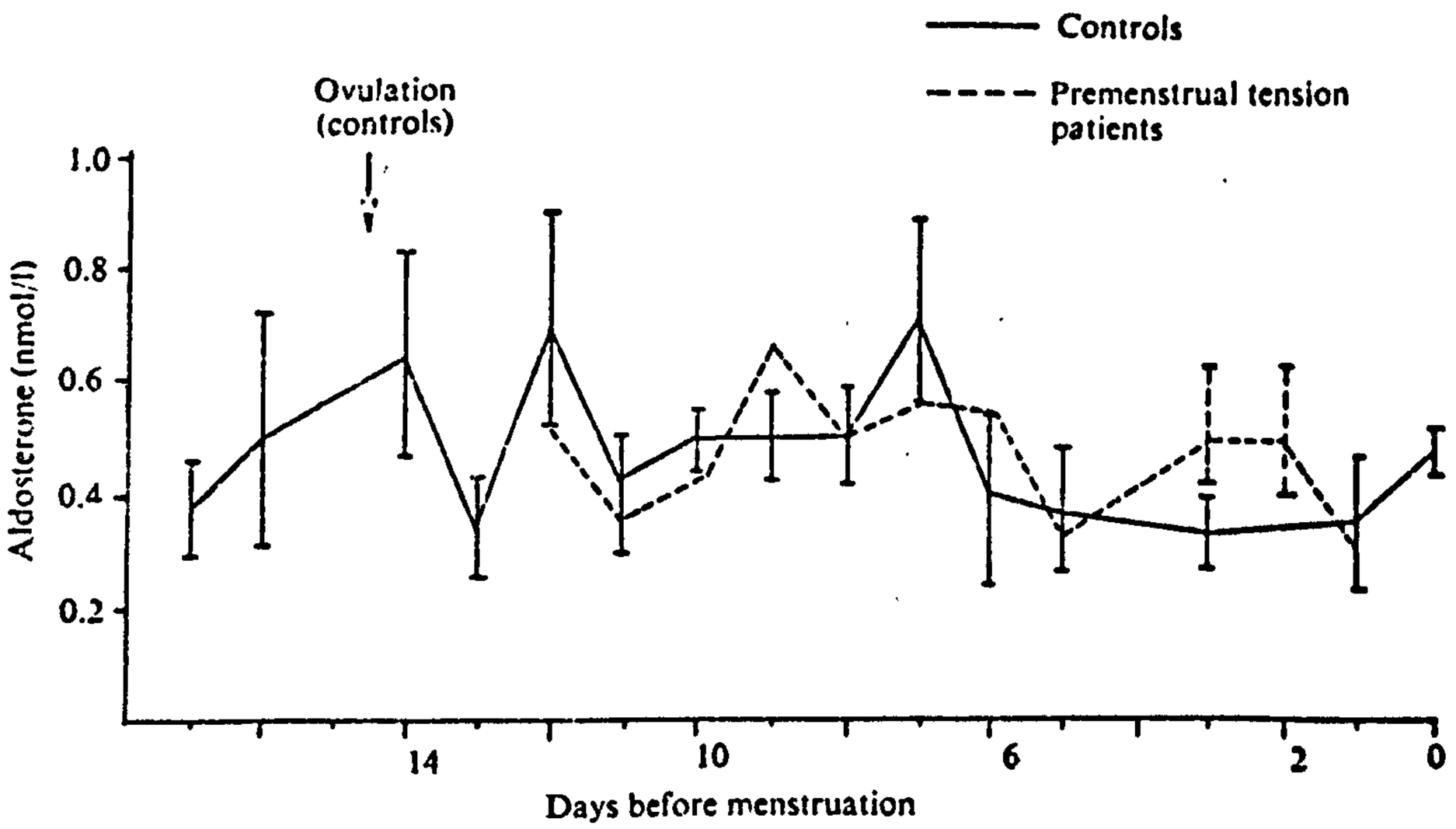
Aldosterone levels were also measured in the 8 premenstrual tension patients. The results are shown in Figure 2. As will be seen, there was a small peak in the controls just prior to ovulation, but the second rise after ovulation was not maintained after the third week of the cycle. This general pattern is in agreement with the results of Frolich *et al.*[4] In the premenstrual tension group there was no consistent

Figure 1. Mean (±S.E.M.) plasma progesterone values in 8 controls and in 8 premenstrual tension patients



Note: days dated backwards from 1st day of menstruation

Figure 2. Mean (±S.E.M.) plasma aldosterone values in 8 controls and in 8 premenstrual tension patients



Note: days dated backwards from 1st day of menstruation

change in aldosterone although there was a small peak in the last 2 days premenstrually. In fact, there was very high variability between individuals and from day to day. There was no difference in aldosterone levels between those who gained weight cyclically and those who did not, but the numbers were very small.

Group 2

A much wider variability of progesterone levels was found in this group of 58 patients than in the smaller first group of patients studied. Patients were sub-divided into those with normal progesterone values and those with values below the 90% confidence limits of the controls (Table III). There was no age difference between the sub-groups but those with low progesterone levels had shorter cycles. Also, the low progesterone level sub-group was larger than one would have expected on statistical grounds from the general population.

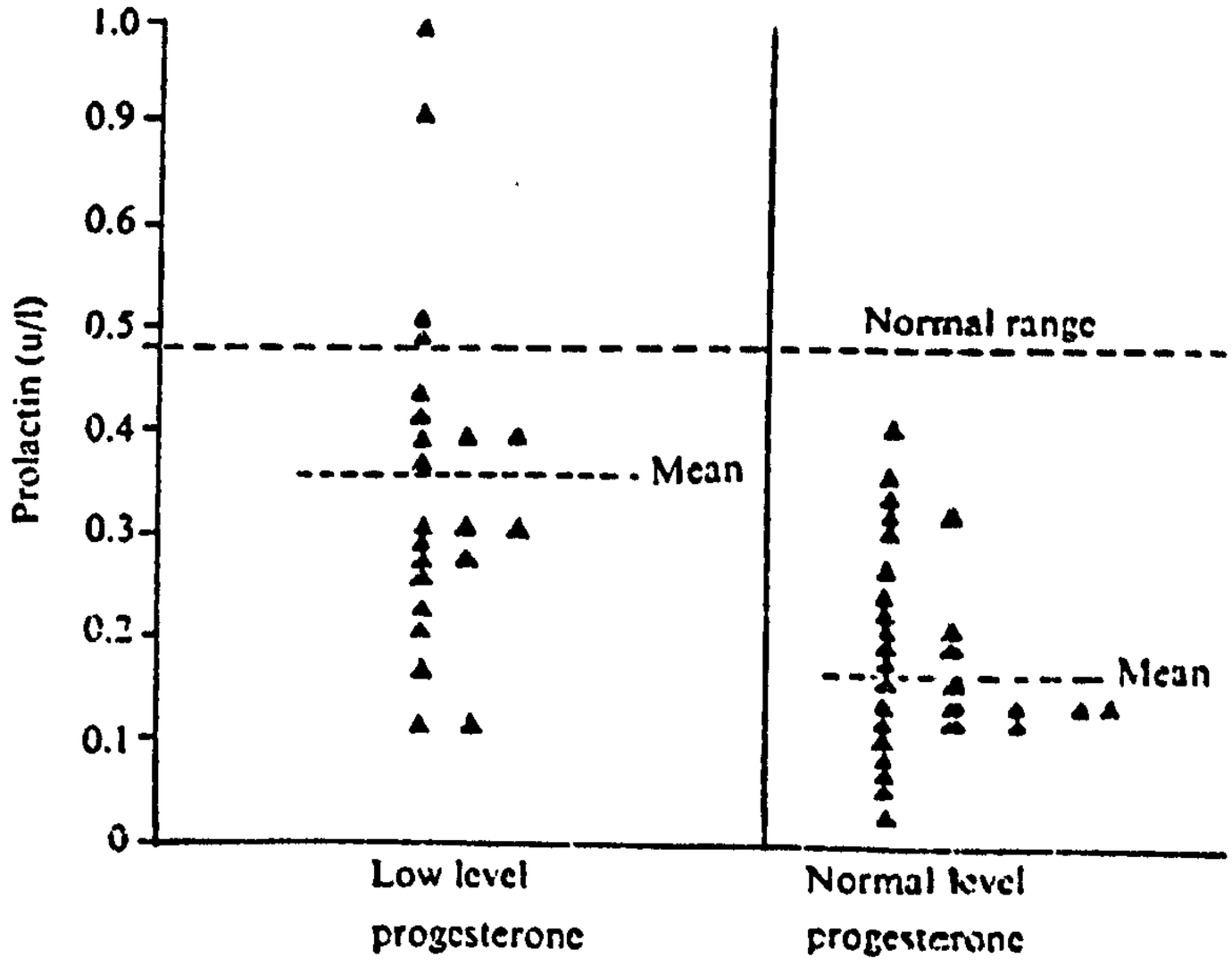
Table III. Progesterone values, age, and length of menstrual cycle in 58 patients with severe premenstrual symptoms: mean values (±S.D.)

Progesterone values	No. patients	Age (yrs)	Length of menstrual cycle (days)
Within normal range	41 (71%)	36 ±5	29 ±2
Below normal range	17 (29%)	34 ±6*	27 ±2**

*not significant **$p<0.02$

Figure 3 gives the prolactin levels of the luteal phase in relation to the progesterone values at the same time in the two sub-groups.

Figure 3. Prolactin levels in sub-groups of premenstrual tension patients with normal progesterone levels (n=30) and with progesterone levels (n=22) below normal (90% confidence limits)



It appears that the low progesterone sub-group tended to have rather raised and variable prolactin results, although only 2 patients had prolactin levels above the normal limits. These results compare with those of Halbreich *et al.*[7] who found raised prolactin levels in premenstrual tension patients compared to controls in the last 3 weeks of the cycle. These investigators, however, gave no reference to normal values.

Discussion

These results show a deficit of progesterone 8 days premenstrually in women with severe premenstrual tension; yet in 6 of the 8 patients studied, symptoms started more than 8 days premenstrually. This suggests some other factor is acting earlier in the cycle, with secondary effects on the corpus luteum. Possible candidates for this factor include prolactin, aldosterone and oestradiol.

It has been shown[17] that the rise in aldosterone excretion in the second half of the cycle only occurs when a corpus luteum is formed. Thus, the natriuresis and diuresis brought about by the anti-aldosterone effect of progesterone[11] is compensated by increased aldosterone secretion. No disturbance in aldosterone plasma levels, however, was found in premenstrual tension patients even when the progesterone levels were low. It seems likely, therefore, that some other factor, either in the renin-angiotensin system or elsewhere, must be responsible for the oedema seen in premenstrual tension. Other workers have found that aldosterone excretion is indeed low in women with cyclical oedema.[14]

The work of Backstrom and Carstensen[1] on a small group of patients suggests that plasma oestradiol is raised in the last 6 days of the cycle. Backstrom and Mattsson[2] have found that oestradiol is raised 3 days premenstrually in patients with psychological symptoms, but not in those who complained of feelings of swelling. It would be interesting to study the levels of oestradiol earlier in the cycle and it is hoped to investigate this in our present patients. Prolactin may also have a role to play in this syndrome. The raised values found in some of our patients, especially those with low progesterone, would seem to agree with some recent evidence that prolactin reduces progesterone production by corpus luteum cells.[12]

It appears that there may be several sub-groups within the large proportion of women who complain of premenstrual tension. In about 30% of the 58 women studied, we found lower progesterone and raised prolactin in the second half of the cycle. We also found that in these women, the slight deficiency of the corpus luteum was reflected in slightly shorter cycles. These preliminary studies suggest that aldosterone has no striking role to play. Further studies are needed to clarify the detailed roles of prolactin and oestradiol in this syndrome.

Acknowledgements

We are indebted to the St. Thomas's Hospital Charitable Trust and the St. Thomas's Hospital Endowment Funds for their financial support. We should like to thank Dr. M. J. Wheeler, Department of Chemical Pathology, St. Thomas's Hospital, for the serum prolactin determinations.

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